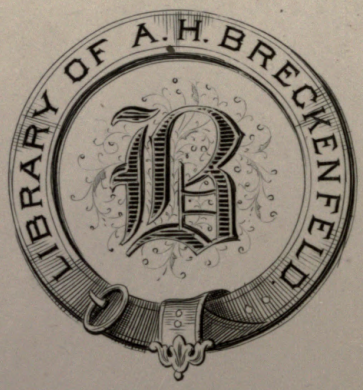
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THE METHODS

OF

BACTERIOLOGICAL INVESTIGATION.

BY

DR. FERDINAND HUEPPE,

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ILLUSTRATED BY THIRTY-ONE WOOD-CUTS.

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DEDICATED IN THANKFUL RESPECT

TO THE

GEHEIMEN REGIERUNGSRATH,

DR. ROBERT KOCH.

AUTHOR'S PREFACE.

URGED by the wish of my highly esteemed teacher, the Geheimrath Koch, I have attempted in the following work to meet the lack of a comprehensive representation of the methods of bacteria-investigation. It was my endeavor, as an historical and experimental critic, to sift the whole of the literature, which was extraordinarily scattered and in part very difficult of access, and to select the good from the hardly conceivable confusion of useful and useless communications, in order to give to the independent investigator a useful hand-book, and to the beginner a trustworthy introduction into this territory.

THE AUTHOR.

WIESBADEN, *February, 1885.*

TRANSLATOR'S PREFACE.

THIS translation of Hueppe's excellent work on the "Methods of Bacteriological Investigation" was suggested by the want in English of a satisfactory text-book on this subject for the use of students working under my direction in the Carnegie Laboratory. The preparation of the original was undertaken by Dr. Hueppe, at the request of Prof. Robert Koch, and the work has been thoroughly and carefully done. It shows a complete familiarity with the subject in hand, is comprehensive in character, and treats carefully all of the approved methods of investigation.

Some difficulties have been met in the translation which those acquainted with this kind of work will readily understand. It is literal so far as is consistent with clearness, and no attempt has been made to attain elegance in style or diction; but it is well known that many German terms have no exact English equivalent, and can only be rendered accurately by a roundabout expression. This is well illustrated by "Massenkultur" (as translated by quantity-culture on page 101 *et seq.*), which means a culture, whether pure or not, where a great

quantity or bulk of bacteria are growing; but, since this expression can not be inserted each time in the text, *quantity-culture* will be used wherever it occurs.

The labor of preparing the translation was performed in the short intervals of leisure found in the midst of numerous other duties, and was unavoidably interrupted just before its completion, so that some errors may be found in the book, but I think that none of serious importance have escaped correction.

The author has requested me to emphasize the fact "that the short, concise form chosen in the work is based on an extensive historical and experimental review of the whole subject, and that if any methods, still much used, have been omitted or only briefly considered, it is because they have not now the significance or importance that has been ascribed to them by other writers."

I here desire to acknowledge my indebtedness to Dr. L. W. Hubbard and Dr. S. N. Nelson, both of New York, for valuable assistance kindly rendered me in the translation.

The work has been very favorably received in Germany, and if this translation meets only a small part of the same consideration in America, I shall feel well repaid for the labor expended.

HERMANN M. BIGGS.

CARNEGIE LABORATORY, NEW YORK,
December 1, 1885.

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BACTERIOLOGICAL INVESTIGATION.

INTRODUCTION.

THE perfection of peculiar methods for the study of bacteria was conditioned, on the one hand, by the minuteness and rapid multiplication of these lower organisms, by reason of which the formerly approved methods scarcely at all sufficed to determine their morphology; and, on the other, by the biological processes in which these micro-organisms take part, and which the methods for their study must also consider.

In regard to their biology, the bacteria may be separated into two great groups: the septic (saprophytic) bacteria, which feed on dead organic bodies, and the parasitic, which are found in living organisms.

Among the septic bacteria, the true bacteria of putrefaction should be distinguished from those which cause a more typical decomposition of lifeless organic matter. In the latter it is possible to even separate chemically the products formed. These last septic forms are designated as ferment bacteria. Aside from these, the pigment bacteria also deserve special notice.

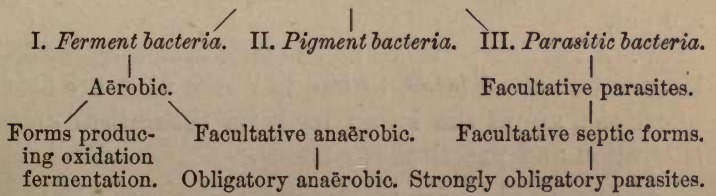
Many of these septic micro-organisms need, under all conditions, the oxygen of the air for life and activity—the aërobic forms ; some species can for the time being be deprived of this, and are able even then to bring about their specific decomposition, but they can also live and multiply with a free access of air. These may be designated as the optional anaërobic (facultativ-anaerobiotische) forms. Again, of other forms it has been asserted that the absence of the oxygen of the air is necessary for their life and activity, and that they are immediately destroyed by oxygen—the obligatory anaërobic (obligat-anaerobiotische) forms. We know of still other forms in which, directly contrary to this, a transference of the oxygen to the medium in which they grow takes place with their life activity. These cause an oxidation fermentation.

Among the bacteria living as parasites, there are forms which do not complete their development upon the animal organism, but only occasionally appear as parasites, or pass through a portion of their existence in living organisms—the facultative parasites of van Tieghem ; other forms find, as a rule, only in living organisms all the conditions necessary for their existence, but can occasionally, or in certain stages of development, also live as septic bacteria—the facultative septic forms of de Bary. Still other forms, finally, seem to be fitted only for the parasitic mode of life, and appear quite incapable of passing any period of their existence as septic bacteria. These are designated as the strongly obligatory parasites of de Bary. Although it is very difficult to determine in individual cases whether a parasitic micro-organism belongs to this or that group, yet in such cases as the recognition of the minute differences also depends

more or less upon the subjective opinion of the observer, it thus permits this grouping to be regarded as much freer from constraint as to their real condition than the division of the pathogenic infectious microorganisms into endogen and ectogen, which has reference only to the extremes. These last designations permit only insufficiently, either a valuation of the investigations concerning the accessory causes of the infectious diseases, or a strong emphasis being placed upon one or another factor, and do not allow the attainment of an unprejudiced judgment upon matters all-important in the ætiology. There is also the greatest difference as to the need of oxygen among the parasitic bacteria. It is to be noted, likewise, that the parasites may be endophytic—i. e., can live in the interior of organs or cells; perhaps also epiphytic—i. e., can live upon the surface.

These different phenomena of adaptability can be theoretically deduced from the simple septic mode of life; but it is to be remembered that direct intermediate links can seldom be determined, and that individual forms can produce different actions.

TRUE SAPROPHYTIC FORMS.



The most general problem which is presented in bacteria investigation—viz., the determination of the group to which a form of bacteria belongs—can now be quickly and accurately solved.

I. It is to be determined whether, in decomposi-

tion or disease, bacteria are present or not. This question associates itself essentially with the question of spontaneous generation and abiogenesis, teaches us the value and general principles of sterilization, and makes us acquainted with the indispensable requirements for reliable work in bacteriology.

II. If bacteria are present, it is to be determined what forms they possess. This general morphological question demands special technical skill, as the ordinary histological *technique* does not suffice.

III. Each form found to be present is to be cultivated by itself, free from all chemical and morphological admixtures—"pure cultures." The problem to be solved by the aid of pure cultures is a double one—i. e., with the help of these the general morphological investigation is completed and extended, and,

IV. By transfers of really pure cultures to decomposable materials or susceptible animals, it is to be determined whether the bacteria found are the cause of the decomposition or disease.

By this investigation it is certainly shown to which of the described groups a form of bacteria belongs; then, extending likewise from the pure cultures, there are yet,

V. A further series of more exact biological problems to be solved later, which, in union with the first questions, afford the broad basis for theoretical consideration and practical treatment.

The solution of all these questions is to be aimed at; but experience has shown that this is not possible in every case, and that cases may come up in which one or the other of these cardinal questions remains unanswered. For instance, as regards the parasitic bacteria which, in the highest degree, represent the

parasitic adaptability, viz., the strongly obligatory parasitic forms, perhaps only the presence of the micro-organism may be determined, while pure cultures can not be obtained. In other forms of this group, a further step has already been taken, by which, to a limited extent, transfers to animals have been made. With individual pathogenic bacteria of the other groups, on the other hand, transfers are not successful, because as yet no species of animals has been shown to be susceptible to the disease which they produce, although pure cultures of these bacteria have been obtained. (Compare Methods of Infection.)

In these cases the greater attention should be given to those problems that can be solved; and it is always to be borne in mind that, with a more complete mastery of the methods in the seemingly most unpromising cases, a classical solution of all questions has sometimes been obtained.

The facts ascertained by means of a perfected *technique* must form, in every branch of natural science, the solid foundation upon which the theories are built up. No investigator, although in possession of many facts, can dispense with the guiding ideas that these afford; but the aversion of many investigators to every speculation is immediately aroused, for the reason that by many the deductions of natural philosophy are stated as scientific facts. Even the subject of bacteriology has in this respect passed through an experience too sad to allow us to forget that the observations in natural philosophy can be nothing else than provisional explanations of phenomena not yet really understood, the value of which, however, in suggesting lines of investigation, is very great. Resolutely must we guard ourselves against

placing under restraint such deductions, often confused, but yet in harmony with facts; against bringing into discredit the solid foundation of facts by cheap badinage of the endeavors of "learned ones" in search of "little facts"; or against citing ever anew agreeable theories or refuted statements as proved facts, as has often happened in the domain of bacteriology. Resolutely also must we see to it that speculations do not form the foundation of the practical treatment of hygiene.

Against such sad errors of speculation there is no better remedy than the familiarity to be obtained with the methods (constantly becoming more accurate) which are so closely united to the real advances in the knowledge and possibilities regarding the causal relation of micro-organisms to decomposition and disease.

I.

SPONTANEOUS GENERATION AND THE PRINCIPLES OF STERILIZATION.*

SPALLANZANI † opened the series of scientific experiments concerning spontaneous generation and the principles of sterilization. He placed infusions of organic substances in flasks, which were corked and sealed, and then boiled for an hour in a water-bath. These experiments, which form the groundwork of the Appert method of preservation of organic substances, can be better made in flasks with long-drawn-out necks, which are closed during the process of heating. Now and then one of these experiments (where the duration of the heating is only one hour) fails. But the chief objection to the conclusions drawn from these experiments lies in the fact that no oxygen can gain admission to the flask. In 1810 Gay-Lussac expressed the opinion that, for the production of fermentation, oxygen must have admission.

The next methodical advance in this direction consisted in the admission of air into the vessel after heating, this air having been previously so treated

* "Zusammenfassende Darstellungen der Frage über Abiogenesis; Generatio spontanea finden sich bei Gscheidlen." "Physiologische Methodik," Heft 2, 1876, S. 274; Heft 4, 1879, S. 499; und bei Tyndall, "Essays on the Floating Matter of the Air," second edition, 1883.

† "Physikalische und mathematische Abhandlungen," 1769.

that it could contain no germs. Franz Schulze * designed a flask which had a double perforated cork, in which were placed two glass tubes bent at a right angle, and which terminated close underneath the cork. The decomposing infusions or substances suspended in water were heated on a sand-bath until all portions had reached the temperature of boiling water. Then, while the steam was still escaping, a Liebig's globe apparatus was fastened to each of the glass tubes. One of the globes was filled with concentrated sulphuric acid, and the other with a solution of caustic potash. Now, by the application of suction-force on the side to which the apparatus containing caustic potash is attached, air is drawn into the flask which before its entrance must pass through the sulphuric acid. When this is done, no decomposition takes place, notwithstanding the presence of the air; but it soon occurs if, after the heating, ordinary air is allowed admittance.

Schwann † showed that "it is not the oxygen, or at least not alone the oxygen, of the atmospheric air" which causes fermentation and putrefaction, by passing the air through mercury which was heated to the boiling-point. No change occurred in the fluids after this was done.

To meet the objection, that by this procedure the air might be altered chemically, Schröder and von Dusch ‡ passed the air through cotton, which was

* "Vorläufige Mittheilung der Resultate einer experimentellen Beobachtung über Generatio aequivoca." Poggendorf's "Annalen der Physik," 1836, Bd. 39, S. 487.

† "Vorläufige Mittheilung, betreffend Versuche über die Weingährung und Fäulniss." Poggendorf's "Annalen," 1837, Bd. 41, S. 184.

‡ "Ueber Filtration der Luft in Beziehung auf Fäulniss und Gährung." "Annalen der Chemie und Pharmacie," 1854, Bd. 89, S. 337.

either placed in tubes connected with the right-angled glass tubes, or they stopped the neck of the flask with cotton during the process of boiling. Pasteur* boiled the infusions in flasks, the necks of which were long-drawn-out and curved in different ways, with only this precaution, that the open end always looked downward. In this way the air could enter, after the heating, unaltered and unfiltered, and no putrefaction took place.

In milk, Pasteur succeeded in preventing decomposition certainly only by elevating the temperature to 110° or 112° C. in a pressure of one and one half atmospheres. Schröder also showed the inefficacy of boiling to prevent decomposition in respect to single substances, which he found could be sterilized with certainty only after a long-continued boiling, or by elevating the temperature in a digester with a pressure of about two atmospheres.

The digester, or steam-kettle, for expanded steam, is used for sterilization with a high temperature. The temperature required for sterilization ranges between 110° and 112° C., corresponding to a pressure of from one to two atmospheres. Since an equalization of temperature is produced by the strong currents in the fluid brought about by the differences in temperature, the best execution, reasoning *a priori*, ought to be expected from this apparatus, since, in accordance with the dynamic theory of heat with the elevation of the temperature, the time required for the equalization of the temperature, and hence the time for sterilization, is diminished. Many

* "Mémoire sur les corpuscles organisés qui existant dans l'atmosphere." "Annales de chimie et de physiques," III. Ser., T. 64, 1862, S. 66. And shorter article, "Compt. rend.," Bd. 48, 1859, S. 337; and ibid., Bd. 50, S. 849.

forms of apparatus* correspond to this theoretical postulate, but all† do not satisfy it in all respects. The further consideration is added to this uncertainty that many substances are chemically altered by the elevation of the temperature above 100° C., so that the apparent advantages of the apparatus, as compared with others, are in many cases quite illusory. Any one who possesses a reliable, tested steam-kettle (Dampfkessel) may safely use this, especially if the substances to be sterilized will bear an elevation of the temperature above 100° C.

If it is desired to use a temperature above 100° C., without employing a steam-kettle, a salt-, oil-, or paraffine-bath can be employed.

If boiling water is to be used, then the larger flasks, smaller flasks, and test-tubes are boiled directly in the water-bath, but the water in it should have a somewhat higher temperature than the contents of the glasses. The equalization of temperature in the water-bath is not only attained correspondingly slower than at a lower temperature, but it is so uncertain that it should be practically tested.

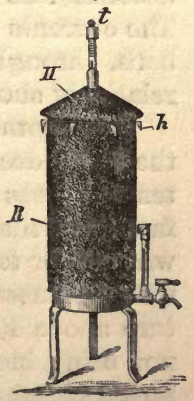
If a sufficiently long boiling affords a real sterilization, then the temperature of the boiling water can be better used in the form of streaming steam. The steam sterilization-cylinder of Koch, Gaffky, and Löffler answers this purpose (Fig. 1). This consists of a cylinder made of strong tin plate, about half a metre high and from twenty to twenty-five centime-

* Fitz, "Ueber Spaltpilzgährungen." "Berichte der deutschen chemischen Gesellschaft," Bd. XVII, 1884, S. 1188.

† Koch, Gaffky, Löffler, "Versuche über die Verwerthbarkeit heisser Wasserdämpfe zu Disinfectionszwecken." "Mittheilungen aus dem kaiserlichen Gesundheitsamte," I, 1881, S. 322.

tres in diameter, which is surrounded by an asbestos covering to prevent the loss of the heat, and is provided with a copper bottom. In the interior at *R*, in the lower third, is placed a grate; the space under this is filled three fourths full of water, which is brought to the boiling-point by a number of gas-flames (three or five burners) placed under the vessel. It is closed above by a cover (*H*) made of block-tin covered with asbestos, and is not hermetically sealed, so that the steam can escape around the edges. In a hole in the cover a thermometer (*t*) is placed.

Fig. 1.



The apparatus may also have somewhat larger dimensions. But, if these dimensions are very much increased, it is necessary to use salt solution in order to keep the temperature of the outer currents of steam at 100° C. If the escape of the steam is not quite free, and the radiation of the heat is prevented, the temperature of the interior of the cylinder in this way may be kept equal throughout; and since the cover is not hermetically sealed, the temperature of the steam does not exceed the boiling-point, but gives the temperature of boiling water, corresponding to the conditions of pressure—that is, with the barometer at nearly its normal height, about 100° C.

The advantages of this apparatus, in comparison with the steam-kettle, are its cheapness and the impossibility of exceeding the temperature of 100° C. when water is used, so that all substances can be sterilized with this, which will bear a temperature of 100° C. The equalization of the temperature is very soon reached, and does not undergo such oscillations

as with the steam-kettle, because the technical use of the apparatus can scarcely be simpler, and on this account this element is reduced to the minimum. The currents of steam are far superior to the water-bath, through the certainty of their action and the relatively short time required.

These practical advantages render this apparatus the most desirable one for all cases in which high temperatures are used for sterilization, in spite of the fact that, theoretically, to compensate for the somewhat lower temperature, it must be used longer than would be necessary for expanded steam at a temperature above 100° C. The beginning of the heating not being included, the time required for the sterilization varies from one half to two hours, depending on the size of the object.

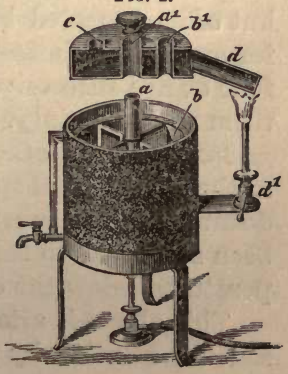
A vessel which fits in the cylinder is included with the apparatus. In this are placed the small flasks or tubes to be sterilized, and to the handle of the vessel or to the neck of the larger flasks a string is fastened for conveniently raising and lowering them. This string is made fast to the hook *h*, to be found on the rim of the cylinder.

But many substances are altered by exposure to a temperature of 100° C., and especially coagulation of the albumen is always brought about. In order to avoid this, the discontinuous sterilization of Tyndall is used. The principle of "Sterilization by Discontinuous Heating" (*l. c.*, pp. 210 and 337) was founded on the observation that living bacteria are killed by exposure to a relatively low temperature, below the point required for the coagulation of albumen, while the spores are not destroyed by these low temperatures,* but are easily killed after germination. If

* Cohn, "Untersuchungen über Bacterien," IV; "Die Bacterien

the fluid to be sterilized is exposed for one or two hours to a temperature of from 52° C. to 65° C., only the living bacteria are in this way destroyed, and perhaps not even all of these the first time. The resistant spores possibly present in the solution thus treated germinate some on the first and second, others on the third and following days. If now the fluid is exposed to the same temperature as before on the second and third days, the living bacteria, or those that have developed from the spores, are killed each time, so that, if this operation is continued long enough, it is possible to sterilize with certainty all fluids below the temperature producing chemical alterations. In general, exposure to a temperature of about 58° C. for one or two hours, on from five to eight successive days, is recommended. This may be done by the use of the water-bath, but more conveniently with the apparatus shown in Fig. 2. This consists of a double-walled cylinder made of copper. The chamber between the two walls is about half filled with water, and the cylinder itself well closed with a double-walled cover, which is also filled with water. The cover has upon its side a hollow tube (*d*), whose lumen communicates with the chamber in the cover. This is warmed by the flame (d^1), while the cylinder itself is warmed from below by a flame placed under it. There are three tubes in the cover, one of which (*c*) is used for filling the cover

Fig. 2.



und die Urzeugung." "Beiträge zur Biologie der Pflanzen," Bd. II, Heft 2, S. 249, 1876.

and for receiving a thermometer which indicates the temperature of the water in it; a second (b^1) receives a thermometer which passes into the air-chamber (b) of the cylinder; and the middle tube (a^1) receives a thermometer which passes into a small central cylinder (a), the cavity of which communicates with the water-chamber on the exterior. The outer water-chamber is filled by means of a tube placed on the side.

The principle of discontinuous sterilization may also be used in many cases as discontinuous boiling, since there are many substances which are altered by long boiling that are not materially changed by brief but often repeated boiling. If such substances—as, for example, gelatin—are boiled for a short time on four or five successive days, it is possible to sterilize them with certainty.

For refuting other objections which are made as to the existence of spontaneous generation, it may be desirable to use substances which have not even once been subjected to the lower temperatures—i. e., temperatures below the coagulation-point of albumen.

This object is attained in two ways: first, by freeing the solution by filtration from possible admixtures; or, secondly, by endeavoring to obtain the same uncontaminated from the beginning.

In respect to the first, Helmholz* observed that the fermentation produced by yeast did not pass through a membrane; on the contrary, this did occur with putrefaction. Consequently such membranes are not available for this purpose. Positive results were first obtained by Tiegel,† who succeeded in separating

* "Ueber das Wesen der Fäulniss und Gährung." Müller's "Archiv für Anatomie und Physiologie," 1843, S. 453.

† "Correspondenzblatt für schweizer Aerzte," 1871, S. 275; und

mechanically—by filtration of septic fluids through clay cells, using positive or negative pressure on one side—the septic material from the quite inactive fluid. Miquel and Benoist* endeavored to remove the germs by a gypsum filter. They took glass balloons with drawn-out necks, in the narrow portion of which an asbestos-stopper was placed, and beyond this a layer of gypsum. Before use the apparatus is slowly heated to 170° C., then the fluid to be filtered is slowly poured upon the gypsum-stopper. The stopper consists of 1·6 asbestos, 52·1 gypsum, 46 water. The diluted juice of flesh and plants and urine filtered rapidly, serum and albuminous fluids somewhat more slowly; all were germ free and free from life. A capillary tube, cemented underneath the constriction, by being united to an aspirator, permits the reduction of the pressure in the balloon.

Gautier† used a very long-necked flask of faïence or unglazed porcelain, which tapered below into a cone. Through this porous cone, the real filter, the fluid to be filtered passed from without into the interior of the porcelain flask. For rarefying the air in the neck of the flask, a glass tube, bent at a right angle, is fastened by vermilion-paint cement, so that the shank reaches down to the bottom of the cone while the other outer end tapers into a small cone and exactly fits into a corresponding conical expansion of a second tube. This second glass tube is likewise bent at a right angle, and the end, which is

"Ueber die fiebererregende Eigenschaft des Mikrosporen septicum." Dissert. Bern, 1871. Citirt nach Klebs.

* "Bulletin de la société chimique de Paris," 1881, Bd. 35, S. 552.

† "Stérilization à froid des liquides fermentescibles." "Bulletin de la société chimique," 1884, Bd. 42, S. 146.

united with the portion of the first tube, possesses a conical expansion, while the other end reaches to the bottom of a glass flask with a narrow neck. To the side of this glass flask a tube with a conical expansion is cemented. The two conical expansions are closed with cotton, and then this glass balloon with its projecting portion and the glass tubes are sterilized. In the same way the porcelain flask with its glass tube is heated, and, after removal of the cotton stopper from the cone of the first glass tube, this is inserted into the conical expansion of the second. In the conical expansion of the projection from the glass flask, after the removal of the stopper, a glass tube, extending out into a corresponding cone, is introduced, which is filled with heated asbestos. The joints uniting the conical expansions with the corresponding conical constrictions are covered with shellac. By aspiration at the free end of the asbestos-tube, the air in the entire apparatus is rarefied, and, when the cone of the porcelain flask is immersed in a fluid, by the existing negative pressure, fluid which is quite free from germs is aspirated into the porcelain flask. The vermilion-paint cement, prepared with oil of turpentine, consists of

Boracic acid (crystallized).....	8 parts.
Silicic acid.....	2 "
Vermilion	12 "

Most of the filtrates obtained free from germs in this way have experienced no material alterations; but some of them do not remain unchanged after the filtration, as the filter does not allow all materials to pass through equally well, and albuminous solutions are often quite seriously altered quantitatively and qualitatively.

In order, also, to eliminate this possible error, the

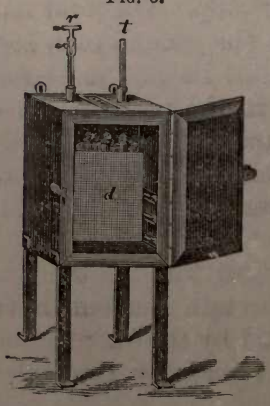
substances should be obtained, quite uncontaminated, by the use of the greatest cleanliness. Before each manipulation the hands are cleansed with a one per cent solution of corrosive sublimate, and are then rinsed in sterilized water, or the sublimate is removed by alcohol, the alcohol by ether, and the latter allowed to evaporate. All vessels are well sterilized; the manipulations and all operations are performed very rapidly and with the antiseptic precautions of modern surgery. In this manner it is possible to obtain blood, milk, urine, etc., without anything ever being mingled with the same, or without decomposition taking place. Some details follow later in the methods of infection and in the inoculation experiments. No organism is ever formed, not even a micrococcus, either from the unorganized material, or from "molecules of nitrogen," or from microscopic forms of life, or from the anamorphosis of protoplasm, which fact certainly has not prevented many authors from mistaking, for real cocci, bodies of different origin showing molecular motion, from which also later, bacilli, etc., should develop. Whoever has obtained contrary results, upon the ground of a few experiments, must first show that he has mastered the *technique*, as was the case in the many positive results of van den Broek, Pasteur, Roberts, Lister, Cheyne, and Meissner. The technical dexterity necessary for this is only to be obtained by many individual experiments.

By these experiments, arranged for spontaneous generation (which, especially through Pasteur, have taken a form easy of mastery), and by the disinfection experiments of Koch, the still remaining principles of sterilization have been reduced to a reliable and convenient form.

Metal objects—scissors, knives, pincettes, platinum-needles—are first mechanically cleaned, then are heated in the flame, and for cooling are laid upon a sterilized glass plate and protected from dust by a bell-jar.

Glass objects—glass plates, slides, flasks, test-tubes—are first mechanically cleaned, then, if they are very dirty, greasy, or have been used for other purposes, are dipped in concentrated sulphuric acid or hydrochloric acid, and finally repeatedly washed in distilled water until every particle of the acid has been removed. The water is first allowed to run off, and the articles thoroughly dried in the dry-oven, or it is removed by means of alcohol, in the following manner: they are dipped into alcohol, and the last particles of this are removed by ether, which is allowed to evaporate. According to the degree of uncleanliness of the vessels in the beginning, either this whole procedure must be gone through with, with the greatest care, or it is sufficient to wash them in distilled water. The *chemically* clean vessels must then be freed from *germs*. For this purpose the vessels, having their necks stopped with a closely fitting mass of cotton, are immediately placed in the double-walled dry-oven (Fig. 3). This is provided with a thermo-regulator (*r*) and a thermometer (*t*). The test-tubes are more conveniently placed in a basket (*d*) made of wire, which will hold a large number. Catheters, syringes, pipettes, capillary-tubes, and other glass tubes are

Fig. 3.



placed in a clean glass and then put in the dry-oven. All such glass objects should remain at least two hours* at a temperature of 150° or 160° C., the time required to raise the temperature to this point not being included. The objects are allowed to cool in the oven, so that they can be removed directly before use, or at least care should be taken to protect them from dust.

Cork stoppers are to be avoided. Rubber corks, caps, and bands, etc., are sterilized in the steam-cylinder for from three quarters of an hour to one hour.

Most vessels with their contents should be sterilized once more; after which it is advantageous to bind over the tops two layers of filter-paper, since the cotton stoppers are proof against bacteria, but not always against fungi.

Previous experience has shown that germs from the air are more seldom the cause of failure than the unintentional infection through unclean or insufficiently sterilized vessels, and the manipulation with hand and instruments not certainly sterilized.

* Practically I have found that exposure to a temperature of 150° or 160° C. for from fifteen to twenty minutes answers exactly the same purpose, and it has been repeatedly shown that neither germs nor their spores will withstand exposure to so high a temperature for this length of time.—Tr.

II.

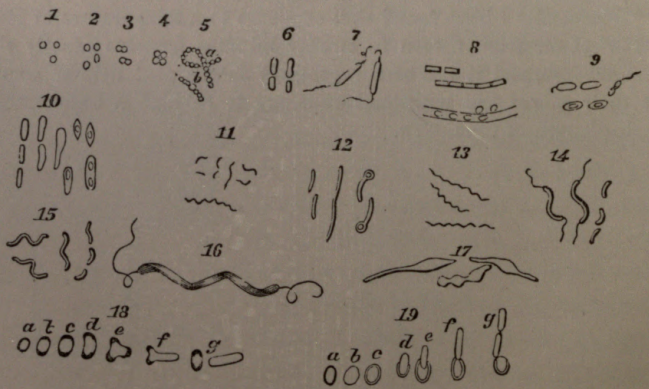
FORMS OF BACTERIA AND MICROSCOPICAL TECHNIQUE.

In the examination, under the microscope, of a substance containing bacteria, different forms present themselves, whose general morphological peculiarities, on the one hand, and whose differences or likenesses, on the other, must be determined.

The forms are determined by the use of the general microscopical *technique*, in its peculiar application to bacteria, while the second question can only be solved after obtaining pure cultures.

The forms of bacteria (Fig. 4) are round (1, 3, 4,

Fig. 4.



In part from Koch and Prazmowski.

and 5), oval (2), shorter (6) or longer (7) rod-form cells; and, in addition, curved rods (11, 12) and spiral-formed organisms (13–16) are observed. These forms appear sometimes isolated, sometimes united in a definite manner (3, 4, 5).

The next object to be attained is the separation of these forms and the grouping of them as naturally as possible, so that a general examination of the morphology of the bacteria may be made.

A. The True Endospore Bacteria (Fig. 4).—These multiply sometimes by division, sometimes also by the endogenous formation of spores.

1. Cocci. { *a*. Round. *b*. Oval. } Cells. } Tendency to the formation of zoöglœa.

2. Straight rods, bacilli. { *a*. Short rods. *b*. Long rods. *c*. Clostridium-forms. } Tendency to the formation of filaments, desmo-bacteria; the filaments show no variation from end to end.

3. Curved rods, vibriones.

4. True spirilla-forms, spirilla, and spirochætæ.

B. Arthrospore Bacteria.—These forms also multiply by division, but they do not produce endogenous spores. On the other hand, single individuals may separate themselves from the colonies and form new generations. These individuals appear almost constantly as round cells, gonidia, or arthrospores.

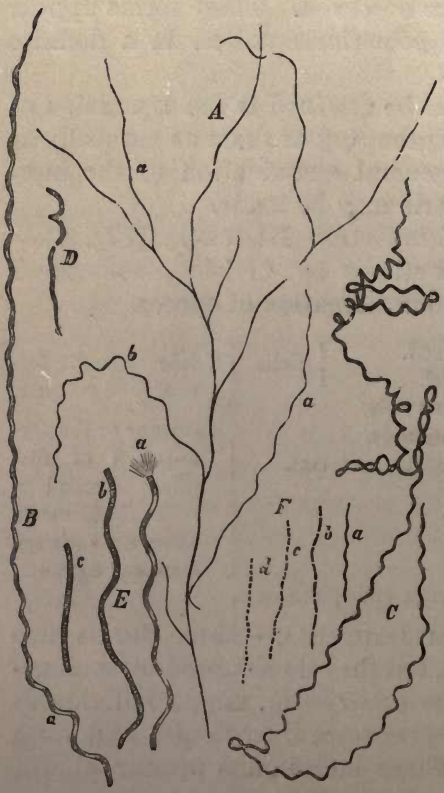
1. *Arthro-Cocci.*—These consist only of cocci, and, by the union of the cocci, form torula.

2. *Arthro-Bacteria.*—These form round cells similar to cocci, but also short rods, long rods, and filaments, which show no variation from end to end.

3. *Leptothrix.*—These form cells similar to cocci, rods, spirals, and filaments, which show a variation from end to end.

4. *Cladothrix* (Fig. 5).—These form cocci, rods, filaments, and spirilla. The filaments form false ramifications (*A*).

Fig. 5.



Cladothrix dichotoma; after Zopf. *A*, branching plant with slightly and decidedly spiral branches. *B*, spiril, one end of which is more winding than the other. *D*, branch with narrow and broad windings. *E*, spirils; *a*, undivided, *b*, divided into rods, and at *c* into Zopf's cocci. *F*, spirochæta form; at *a*, undivided, at *b*, schematic division into long rods, at *c*, into short rods, and at *d* in cocci.

The arthro-cocci and arthro-bacteria have as yet been very little studied, and are perhaps to be classed with the true bacteria. These two groups, so far as they have been studied, seem to stand nearer to the fission algæ on account of their form. Zopf* classes them directly with the bacteria, because of the appearance of forms similar to these in their development.

The next question in the microscopical investigation is to determine which of the described forms are, in general, present. After obtaining a pure cult-

* Compare my criticism of the work of Zopf in the "Fortschritten der Medizin," 1883, No. 6.

ure, it must then be determined what is the typical form; whether it remains the same under all conditions, so that it not only has the value of growth-form, but also constitues a form-species or form-genus; or whether it corresponds to external conditions, being sometimes larger and broader, sometimes smaller and thinner; or whether, finally, other forms may appear in their development. Further, it ought to be ascertained whether, in the development of a species, a single form or a form-cycle appears or can appear.

In regard to the individual peculiarities, there remain yet the following points to be noted, although those already referred to seem to be numerous. In the spiral forms, Fig. 4 (13, 14, 15, 16), the number and the arrangements of the typical spirils should be observed; then it is to be noted whether, in division, the spirils separate into curved rods similar to the vibriones, and whether these fissions-products again develop into typical spirils, or whether the division extends on to the production of forms which are similar to the rod-bacteria and cocci, which finally originate new generations. If reagents are used, it is first necessary to note the stage of development, since, either during the process of division or immediately before it, the reagents will make visible the lines of division in place of the apparently homogeneous character of the filaments. On the other hand, the value of such chemical action should not be overestimated, since, in the height of their development, that most susceptible reagent, photography, shows not the slightest trace of union.

In the case of the vibriones, Fig. 4 (11, 12)—the smallest forms of which (11) Koch, on account of

their characteristic appearance with the usual magnifying power, called comma-bacilli—it should be noticed whether they divide into smaller vibriones, or whether they can form spherical and rod-shaped forms. Through the union of a number of vibriones, S-forms and long, slender filaments similar to spirilla (11) may be produced which the inexperienced observer can easily confound with the true spirilla (13). These spiral filaments, according to the degree of curvature of the individual vibriones, form sometimes slightly wavy lines, sometimes spirils with narrow windings. The spiral forms of the vibriones are to be considered as a kind of thread-formation, which, as contrasted with the true spirilla, appear only under certain conditions, as, for example, the partial or total exhaustion of the culture-medium, and never show the regularity of the latter.

In the rod-formed bacteria it should be observed whether the division of long rods into short ones, or into oval and round cells, predominates. Some rod-forms, by their union, produce long filaments, while others show a tendency to the formation of zoöglœa. On account of the greater tendency of the short-rod bacteria, like the cocci, to form zoöglœa, Cohn separated them from the vibriones and long-rod bacteria, which he classifies as filament-bacteria. It is to be noted whether the character of the culture-medium (solid or fluid) has any influence upon this.

In the cocci it should be determined whether the oval cocci produce, by their division, smaller oval cells or round cells, and whether the round cocci, before their division, become oval, and, finally, whether the oval cocci become short rods previous to their division.

The appearance of straight rods may be produced

in the curved rods, when the concavity or convexity of the rod is turned upward. A straight rod standing upright may present the appearance of a round cell, and a long rod placed at an angle to the plane of vision may appear like a short rod. It is only possible to decide such questions after many single observations and with the use of pure cultures. Many hundred observations of mixtures of bacteria do not serve at all for the decision of morphological questions.

The consideration of spore-formation, Fig. 4 (5 *b*, 8, 9, 10, 12), and germination shows two different types. The rods may develop into filaments before the formation of spores (8), or the spores may appear in the mobile rods (9); sometimes they are formed in the interior (8, 9), sometimes near the end (12). The rods often become whetstone- or club-shaped before the formation of the spores (clostridium, 10). Under other conditions, quite abnormal, so-called involution forms are observed (17).

The work of Cohn * is fundamental as regards the value of form. In this he first clearly described the relations of the growth-form, the form-genus, form-species, true varieties, form-varieties, and physiological varieties. This work is not only often entirely misunderstood by his opponents, but also construed in an opposite sense by his own zealous supporters, who have repeatedly interpreted his arguments for the existence of true species among the bacteria in the sense of a belief in constancy in species and form,† which was accepted before the Darwinian theory.

* "Untersuchungen über Bakterien." "Beiträge zur Biologie der Pflanzen," Bd. I, 2. Heft, S. 127, 1872. 2. Abdruck, 1881.

† For the further investigation of these questions, consult Naegeli,

Determination of the Presence of Bacteria Unstained.

The oldest method of examining unstained bacteria consisted in mingling a small drop of fluid or a particle of matter containing bacteria, with a drop of indifferent fluid, placing it upon a slide, putting over it a cover-glass, and then examining it according to the method of histological procedure.

The picture presented in these cases is of the same nature as in unstained tissue-preparations, especially in this: that the objects, because of their different power of refracting light from the inclosing media, or, according to Koch, "on account of the refraction of the rays of light passing through, present a picture of lines and shadows—viz., the structure-picture."

In these cases a diaphragm is used, as in other histological work in which it is desired to make out the structure-picture.* The diffused daylight will

"Die niederen Pilze in ihren Beziehungen zu den Infectionskrankheiten und der Gesundheitspflege," 1877. Naegeli und Buchner, in Naegeli's "Untersuchungen über niedere Pilze," 1882. Koch, "Zur Aetiologie des Milzbrandes," "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. I, 1881, S. 49. Gaffky, "Experimentell erzeugte Septicaemie mit Rücksicht auf progressive Virulenz und accommodative Züchtung," ibid., S. 80. Flügge, "Fermente und Mikroparasiten," 1883, und "Deutsche med. Wochenschrift," 1884, No. 46. Zopf, "Die Spaltpilze," 2. Aufl., 1884. De Bary, "Vergleichende Morphologie und Biologie der Pilze," 1884. Hüppe, "Fortschritte der Medizin," 1883, No. 6, und 1884, No. 6 (Kritik der Ansichten von Zopf), und "Ueber die Zersetzungen der Milch und die biologischen Grundlagen der Gährungsphysiologie," "Deutsche med. Wochenschrift," 1884, Nos. 48–50.

* Zur weiteren Orientirung über das Mikroskop und die mikroskopische Technik: Dippel, "Das Mikroskop," 2. Aufl., I, 1882–83; Frey, "The Microscope and the Microscopical Technique"; Strass-

not suffice to illuminate the object when the higher powers are used, so that it is necessary to employ a condenser, which does not define the structure-picture, but serves to increase the illumination. For this kind of microscopical work the achromatic condensers of the larger English instruments answer the purpose better than any others.

The dry system of objectives does not suffice for most bacteria; in order to make out only approximately the correct forms, one must use in this investigation the immersion system, in which, according to Amici, the stratum of light, by passing through a stronger refracting medium, compensates as much as possible for the error caused by the dispersion of the rays by the cover-glass.

Since the cover-glass and the front lens of the objective consist of crown-glass, and water, on account of its low refractive power, does not quite correct the error, in the water-immersion systems correction-adjusters and cover-glasses of a certain thickness are necessary.

But the correction is obtained if the immersion-fluid has the same exponent of refraction as crown-glass. Such a fluid, according to Abbé,* presents an optical homogeneous union between the preparation and the objective, which prevents all refraction of the rays in front of the convex surface of the optical system. By this means the loss of light through reflection at the natural joints of the different optical media is avoided, and at the same time a very con-

burger, "Das botanische Practicum," 1884; Friedländer, "Microscopical Technique."

* "Ueber Stephenson's System der homogenen Immersion." "Sitzungsberichte der Jenaischen Gesellschaft f. Med. und Naturw.," 1879, 10. Januar.

siderable amount of spherical aberration is prevented. On account of this, the correction-adjusters necessary in the water-immersion systems can be dispensed with, and the thickness of the cover-glass is of no great moment, since, as soon as the intervening medium has the same index of refraction and dispersion as the cover-glass, the same result for optical purposes is obtained whether a thick layer of glass and a proportionally thin layer of fluid or the reverse is interposed between the object and the lens system.

Anise-oil was used by Amici for the purpose of increasing the exponent of refraction; by Spencer glycerin was employed. Stephenson * desired an enlargement of the aperture of the lens, not only to avoid the necessity of the correction of the cover-glass, but also to increase the power of differentiation.

The union of these two postulates by Stephenson, their settlement by Abbé, the construction of lenses by Zeiss, and the introduction of this system for homogeneous immersion by Koch,† marked a new era for the microscopic side of bacteria investigation.

The best fluid is the ethereal oil of cedar, the index of whose refraction is the same as crown-glass, and its index of dispersion differs only in a slight degree from crown-glass. When somewhat inspissated, it is more convenient for use for most optical purposes. By mixing other stronger-refracting ethereal oils—such as oil of cloves, phenol, and anise—with olive- or

* On a large-angled immersion objective. "Journal of the Royal Microscopical Society," 1878, p. 51.

† "Untersuchungen über die Aetiologie der Wundinfectionskrankheiten," 1878.

castor-oil, an immersion-fluid can be made, which is similar in its refracting power to oil of cedar, or which differs from it to a certain degree.

In consequence of the removal of the troublesome cover-glass correction (attained by different lengths of the draw-tubes) which allows, in a delicate manner, a compensation for the influence upon the aberration of the varying distance of the picture, the objectives for homogeneous immersion are always adjusted for a definite length of the draw-tube. On this account it is to be noted that lengthening the draw-tube beyond this normal length acts in the way of a spherical over-correction, shortening in the way of an under-correction.

In using an immersion-lens, one places a drop of oil on the cover-glass, screws down the draw-tube with the coarse adjustment, or, if this is lacking, brings it down with a rotary motion by the hand, so far that the front lens of the objective touches the oil and the picture begins to be visible. Then the fine adjustment with micrometer-screw is used. Some place a drop of oil on the front lens of the objective. Others put a drop not only upon the objective, but also upon the cover-glass.

After use, the oil is carefully removed from the lens with a fine linen cloth, less satisfactorily with blotting-paper, and the system is returned to its case. If the cover-glass preparation is to be preserved, then the oil is soaked up with filter-paper, and what remains is finally removed by chloroform or benzine.

According to histological tradition, which influenced the earlier use of the microscope, the magnifying power should be increased rather through the use of a more powerful lens than a more powerful

ocular. But, according to a purely physical principle, the strength of an ocular, which an objective will allow with advantage, depends upon the angle of aperture of the latter. The larger the angle of aperture, so much the stronger, other things being equal, can the ocular be. Our best homogeneous systems correspond to this requirement, namely, that one can use the same objective and at the same time employ the most powerful oculars.

In this way, bacteria without any special preparation may be observed between the slide and coverglass. One cause of uncertainty is here noticed, i. e., almost all bacteria are in motion. This in part seems to be spontaneous motion; in part a simple Brownian molecular movement, such as occurs in all fine particles suspended in a fluid. In proportion to the minuteness of the objects, these movements render exact observation difficult. For this reason one should early eliminate this, and fix the forms of micro-organisms by narcotizing them.* For this purpose, a particle of spirituous or dilute alcoholic tincture of opium can be added with the point of a needle to a drop of water.

Moreover, von Recklinghausen† has shown that small, round, granular tissue-detritus, which is so easily confounded with bacteria, can be sharply differentiated by the fact that bacteria are pre-eminently homogeneous granules, and are totally unaffected by the action of acetic acid, glycerine, or even caustic soda.

Baumgarten‡ succeeded in making the tubercle

* Perty, "Zur Kenntniss kleinster Lebensformen," 1852, S. 13.

† "Verhandlungen der Physikal-Medizin. Gesellschaft in Würzburg," N. F., II. Bd., Heft 4, 1872. "Sitzungsberichte," S. XII.

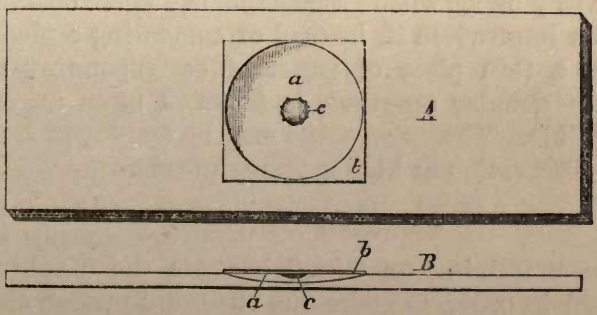
‡ "Centralblatt f. d. med. Wissenschaft," 1882, No. 15.

bacilli visible by their resistance to a diluted solution of caustic soda, though he could not recognize them by staining according to the methods in use at that time.

We possess now more convenient means, both for fixation and differentiation. But it would be a serious error not to examine bacteria unfixed and unstained. It is, on the contrary, necessary throughout that the bacteria should be observed under the most natural conditions possible in order to study their motion, to follow the formation of spores and their germination, and to control the forms according to other treatment.

For this purpose we do not use the previously described form of investigation, but employ the moist chamber. For this the hollow slides *A* and *B* (Fig. 6) serve. A small drop (*c*) of the bacteria-containing

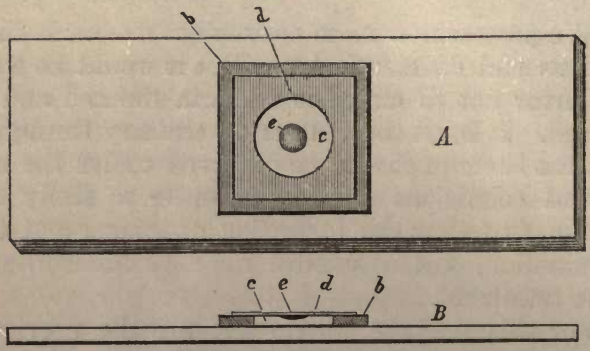
FIG. 6.



fluid is placed upon a cover-glass (*b*). The cover-glass is quickly reversed, and, with the drop now hanging underneath (*c*, *B*), is laid over the hollow (*a*) in the slide, and its edges are surrounded by vaseline, wax, paraffine, or balsam, in order to prevent evaporation of the fluid. Another and a better form is represented in Fig. 7. Upon a slide, *A* or *B*, a glass plate (*b*)

is cemented, which has a central circular opening. A chamber is formed by laying a cover-glass (*a*) over

Fig. 7.



the opening. This room, in place of the almost half-circular cavity in Fig. 6, is bounded by parallel walls. The drop is prepared in a similar manner, and in the same way hangs within the chamber. These chambers can be improvised if, instead of cementing a piece of glass, a thin piece of paper of corresponding size, with a circular aperture, is fastened upon an ordinary slide. The warm stage may be employed for direct observation at higher temperatures.

Staining Bacteria.

In examining unstained bacteria the diaphragm is used in order to make the structure-picture clear; but small objects and particles, the size of bacteria, imbedded in the tissue made visible in the structure-picture, are hidden by the shadows of the structure-picture. If these particles are stained, and if they are of a certain size, they will be visible in spite of the shadow; but under this size, notwithstanding their color, they are concealed by the

shadows. Therefore it is desirable to stain the bacteria and to so arrange the light that the structure-picture does not further interfere, and that the color-picture, as pure as possible, be presented to the observer. Koch (*loc. cit.*) succeeded in producing this isolation of the color-picture by removal of the diaphragm. In this way so weak a structure-picture was produced that the minutest particles of the color-picture became distinct. In the stronger structure-pictures, after the removal of the diaphragm, he used a condenser which threw so intense a cone of rays upon the object that the diffraction appearances were entirely avoided.

With such a method of illumination, in which the preparation is permeated in all directions by the penetrating rays, only those elements remain visible which produce an absorption of the rays on account of their staining. Further, Abbé in this way has shown that, although the illumination in name remains central, yet the important advantages of the oblique illumination are obtained through the co-operation of the rays passing at a greater inclination to the axis of the microscope. On account of this co-operation of the oblique rays for the isolation of the color-picture, and for the complete development of the capacity for differentiation of the oil-immersion objective necessary for this, the condenser must furnish a cone of light of a size at least equal to the aperture of the objective, which is made, according to Stephenson, with a large angle of aperture. This is as yet attained, in a manner that fulfills all the requirements, only by an Abbé condenser. In order that the structure-picture can be brought out in spite of this condenser, an arrangement for interposing a

diaphragm is added, which is furnished with openings of different sizes.

An Abbé condenser belongs to the system for homogeneous immersion in bacteria investigation. Now and then it is useful to place a drop of water or immersion-fluid between the condenser and the underside of the slide, so that below and above a continuous union is formed. These form immersion-condensers which were formerly used before the Abbé condenser was constructed.

GENERAL PRINCIPLES OF STAINING.

Since Hartig, in 1854, and Gerlach, in 1858, showed, by the systematic use of carmine in histological work, that in employing coloring-matters certain elements of the tissues become more distinct and can be differentiated from other elements, staining has been recognized as equivalent to a chemical reaction.

Weigert* first succeeded in staining the zoöglœa masses of the micrococci with the nuclei, by the use of the nuclei-staining ammoniac-carmine solution, and the subsequent treatment with hydrochloric-acid glycerine. This staining, it is true, was first used by Weigert as a staining of cement substance; but later it was corrected by him, and it was in this way shown that the bacteria can be brought into view by other characteristics than their greater resistance to acids and alkalies.

In the following year Eberth and Wagner succeeded in staining micrococci, but not bacilli, with hæmatoxylin.

* "Ueber Bakterien in der Pockenhaut." "Centralblatt f. d. med. Wissenschaft," 1871, No. 49.

Then Weigert* showed that the micrococci, especially in zoöglœa masses, can be stained by different nuclei-staining materials. For this purpose he used at that time methyl-violet, an aniline-dye which stains nuclei. By the subsequent treatment of preparations stained with hæmatoxylin (in which the micrococci and nuclei are stained blue), with diluted caustic potash and strong acetic acid, he first succeeded in procuring an isolated staining of the micrococci. Weigert† further observed that the larger bacilli, which were not stained by hæmatoxylin, could be made visible by certain aniline-dyes. Soon after this, Koch‡ found that the bacteria take up aniline-dyes with such certainty, and so quickly and completely, that "these dyes can be used as reagents for differentiation of bacteria from crystalline and amorphous precipitates, or from the smallest fat-drop or other minute bodies."

Koch# then succeeded in obtaining the isolated staining of the bacteria by washing out the section with a solution of carbonate of potassium, by which all the elements except the bacteria were decolorized. Finally Weigert‖ obtained a double staining, when he subsequently treated with picro-carmine preparations that had been stained with a blue aniline-dye, whereby the bacteria appear blue and the nuclei red;

* "Sitzung der Schlesischen Gesellschaft für vaterländische Cultur," vom 10. December, 1875.

† "Berl. klin. Wochenschrift," 1877, Nos. 18–19; und "Berichte über die Münchener Naturforscherversammlung," 1877, S. 283.

‡ "Verfahren zur Untersuchung, zum Conserviren und Photographiren der Bakterien." "Beiträge zur Biologie der Pflanzen," Bd. II, 3. Heft, 1877, S. 399.

"Wundinfectionskrankheiten," 1878, S. 39.

‖ "Zur Technik der mikroskopischen Bakterienuntersuchungen." Virchow's "Archiv," 1881, Bd. LXXXIV, S. 275.

and Koch* observed that certain forms of bacteria stained differently from the nuclei and from other bacteria which were present in the same preparations.

What coloring-matters ought to be used for staining bacteria?

Weigert's observation that the micrococci, but not the bacilli, are stained by carmine, and a similar observation by Eberth and Wagner concerning hæmatoxylin, seemed to show, according to Weigert, that there are essential chemical differences between the single-group forms of Cohn. Safranine, one of the best reagents for the staining of nuclei, is also of more value for micrococci than for other forms of bacteria. Further, Obermeyer† observed that the spirilla are less resistant to the action of acids and alkalies than other bacteria. But these differences are not essential, since some micrococci and bacilli are less resistant to the action of alkalies and acids than others, and since some bacilli stain as well with hæmatoxylin as micrococci, while others take this dye badly.

However, the basic aniline-dyes have shown themselves to be available staining materials, under all conditions, both for the dried cover-glass preparations and for sections, so that we assign to them a first rank among materials for staining bacteria, and give to other dyes a second place.

Ehrlich,‡ partly in conjunction with his pupils Schwarze and Westphal,# undertook to classify the

* "Berl. klin. Wochenschrift," 1882, No. 15.

† "Berl. klin. Wochenschrift," 1873, S. 391.

‡ "Zeitschrift für klin. Med.," Bd. I, 1880, S. 553; und kleinere gelegentliche Mittheilungen.

Schwarze, "Ueber eosinophile Zellen," Dissert., Berl., 1880. Westphal, "Ueber Mastzellen," Dissert., Berl., 1880.

dyes used in microscopic work. The principle underlying this theoretical study of dyes rests on the observation that the different elements of the tissues and cells possess the capacity of taking certain dyes only, or of holding them with a greater tenacity than other elements. This "election," this affinity of the dyes for certain elements, lends to staining the value of a chemical reaction; or, more correctly (in the want of a *precise* chemical reaction), shows to the eye the presence of differences otherwise imperceptible or distinguishable only with difficulty.

Many staining materials color at first many elements of a tissue quite diffusely, so that the individual elements are not recognizable. If, then, certain decolorizing agents are used, some of these individual elements give up their color, while, on the other hand, other elements retain it persistently. In this way, by an indirect method, it is possible to attain a maximum staining of certain elements, while others remain as much as possible uncolored. Ehrlich called this method, which was first used in another way by Friedländer,* "the principle of the maximal decolorization."

Histologically, one must differentiate in every coloring-material two peculiarities: first, the affinity to certain elements; and, secondly, the staining power. In respect to the election or affinity for certain elements, Ehrlich divides the aniline colors into two groups—(*a*) the acid, (*b*) the basic aniline-dyes—according as the coloring principle is the acid or base-color. In this histological sense it is of equal import whether the acid in its use acts as a free acid or a salt; also whether the base acts as such or as a salt.

* "Studien über automische Herzbewegung," in "Untersuchungen aus dem physiologischen Institut zu Würzburg," I, 1867.

The acid aniline colors are divided into four classes:

1. Fluorescin—e. g., fluorescin and eosin.

2. Nitrogenous bodies—e. g., martius yellow, picric acid, and aurantia.

3. Sulphuric acid—e. g., tropæolin.

4. Primary dye-acids—e. g., rosol acid, alizarin, and purpurin.

Of the basic aniline-dyes, the following are found to have the most value: Fuchsin (muriate of roseanilin), methyl-violet (muriate of trimethyl rose-anilin), gentian-violet, methyl-blue, and vesuvin. Of less value are methyl-green, cyanin, safranin, magdala, and dahlia. Of these, especially the violets (methyl-violet, gentian-violet, iodine-violet, and dahlia) have sometimes the valuable capacity of double staining; that is, they stain certain elements in a color varying from the fundamental color—e. g., methyl-violet does not stain the amyloid substance violet, as it does the bacteria and nuclei, but red; methyl-green stains the nuclei green, the amyloid substance violet.

The members of the first group show altogether the same elective peculiarities—i. e., they act as acid dyes and stain all the accessible elements, but in a differing degree. The basic aniline-dyes also show the same elective peculiarities, since they stain the accessible elements in a basic color; but also in these there is a difference in the intensity of the staining. This intensity of the staining is dependent upon the coloring power, and the coloring power is conditional on the fact that the different coloring-matters are retained in different degrees of intensity in the tissues or cell-elements, in the presence of the individual groups of decolorizers, such as alcohol, acetic acid, and glycerine. For example, methyl-green in a short

time is completely extracted from a preparation by alcohol, while vesuvin is scarcely at all affected. In respect to this coloring power, the basic aniline-dyes arrange themselves in the following order: Vesuvin, bismarck-brown, and aniline-brown theoretically take the first place, because these coloring-matters are not extracted by glycerine, and they are at the same time suitable colors for photography. After these follow in a descending scale fuchsin, methyl-violet, gentian-violet, and methyl-blue; but these should be placed generally before the brown, as they are to most persons more agreeable and satisfactory colors. The remaining dyes have as yet found no general use.

This scale, as I will remark, to avoid misunderstanding, has a conditional value, since certain colors are produced only when certain fluids are used for solution and when the preparations are subsequently treated in a special manner, as will be described later more in detail. At the same time it is not impossible that some one of the remaining dyes may prove to be more valuable than these.

The basic aniline colors are soluble in water, and for the most part in one or all of the decolorizing agents. In use, a weak watery solution colors at first the intercellular substance and the cell-body, while the nuclei remain unstained. Through the subsequent treatment with alcohol, glycerine, or acetic acid an inversion of the staining takes place, by which the elements previously colored become colorless, while the previously colorless nuclei are stained. In the use of the stronger solutions the staining follows (without any discernible inversion) directly and quickly; and, in general, its intensity is in proportion to the concentration of the solution. In a quite concentrated watery solution overstaining may occur, which

in sections can be reduced to the proper degree by subsequent decolorization.

Methyl-blue alone does not overstain, according to Ehrlich,* even after a long action, and it is consequently to be used if for a special reason no decolorizing agents should be employed.

If the dyes are dissolved in the decolorizing agents —such as absolute alcohol, acetic acid, or thick glycerine—they stain slightly or not at all. Instead of using some decolorizing agent subsequently, to reduce the intensity of the staining to the proper degree, in preparations which have been overstained in watery solutions, in many cases a solution of the dye-stuff in a mixture of water with alcohol (Herrmann), glycerine (Schaefer), or acetic acid (Ehrlich) may be used.

PREPARATION OF STAINING FLUIDS.

The basic aniline-dyes are used in the following solutions:

1. Concentrated watery solutions.

These are either used directly or after dilution to the desired degree with distilled water. The solutions are prepared with distilled water (which has been previously boiled), so that an excess of the coloring-matter remains undissolved. They must be often filtered. Only a small quantity of these watery solutions should be made at a time.

2. Concentrated alcoholic solutions.

The solution of an excess of the coloring-material is brought about in the best way by absolute alcohol, or, in want of this, by the officinal 90 per cent spirit of the Pharmacopœia.

In general, one can calculate about 20 to 25 grammes of the dye-stuff to 100 grammes of the spirit

* "Zeitschrift f. klin Med.," Bd. II, 1881, S. 710.

or alcohol. These solutions are kept prepared, and are not used directly for staining, but are mixed with a certain amount of distilled water. In place of concentrated watery solutions, these can be used if five or six drops are added to a small watch-glass of distilled water. This mixture I shall briefly designate in the future as the diluted alcoholic solution.

3. Vesuvin, bismarck-brown, and aniline-brown can not be used in alcoholic solution, nor in a watery solution, even if filtered each time; so that a concentrated solution in equal parts of glycerine and water is prepared.*

4. Alkaline solutions.

a. Weak. Koch.†

Concentrated alcoholic solution methyl-blue..1 c. cm.
Aq. destil.....200 c. cm.
10 per cent solution caustic potash.....0·2 c. cm.

b. Strong.‡

Concentrated alcoholic solution methyl-blue.30 c. cm.
Solution caustic potash, 1 to 10,000.....100 c. cm.

5. Aniline-water solution (according to Ehrlich).#

Pure aniline-oil in excess is shaken with distilled water for one half to one minute (about 5 c. cm. of oil with 100 c. cm. of water). Then, after allowing it to stand five minutes, the mixture is filtered through a filter which has been previously moistened with distilled water. The filtrate must be perfectly clear, and serves in place of water as a menstruum. Since this saturated aniline solution very quickly becomes un-

* Koch, "Verfahren zur Untersuchung." "Beiträge zur Biologie der Pflanzen," 1877, Bd. II, 3. Heft, S. 5.

† "Berl. klin. Wochenschrift," 1882, No. 15; "Mittheilungen aus d. k. Gesundheitsamt," 1884, Bd. II, S. 5.

‡ "Mittheilungen," 1884, Bd. II, S. 439.

"Deutsche med. Wochenschrift," 1882, No. 19.

stable, it is better to prepare it fresh each time that it is used. If it is desired to make this permanent, according to B. Fraenkel, 5 to 10 per cent of alcohol is added, or 3 c. cm. of aniline-oil is dissolved in 7 c. cm. of alcohol and 90 c. cm. of distilled water is added. Fuchsin, methyl-violet, and gentian-violet are the best dyes for use in this menstruum.

In most cases, according to Ehrlich, it is more convenient to add a saturated alcoholic solution of fuchsin or methyl-violet to the clear aniline-water until a distinct cloudiness of the fluid is present, which indicates that the fluid is saturated with the coloring-matter. For certain purposes the following modification of the Ehrlich solution, according to Weigert and Koch,* recommends itself for common use, but this must be renewed after ten or twelve days, because its coloring power is generally diminished:

Saturated aniline-water.....100 c. cm.
Concentrated alcoholic solution, methyl-violet, or fuchsin..... 11 c. cm.
Absolute alcohol..... 10 c. cm.

6. In place of aniline, toludin, prepared in the same manner, can be used as a menstruum (B. Fraenkel).† Also turpentine (Prior);‡ and a five per cent watery solution of carbolic acid (Ziehl),# or one half per cent ammonia (Weigert)‖ [Liq. ammon. caust., 0·5 c. cm.; aq. destil., 90 c. cm.; alcohol abs., 10 c. cm.; gentian-violet, 2 grm.].

For double staining, the nuclei-staining carmine

* "Mittheilungen aus dem kaiserlichen Gesundheitsamt," Bd. II, 1884, S. 6.

† "Berl. k. Wochenschrift," 1884, No. 13.

‡ "Berl. k. Wochenschrift," 1883, No. 33.

"Deutsche med. Wochenschrift," 1882, S. 451; 1883, S. 12 und 247.

‖ "Deutsche med. Wochenschrift," S. 351.

and hæmatoxylin* can also be used; the first for blue or violet, the latter for red-stained bacteria. In place of the ordinary nuclei-staining carmine, picrocarmine can be used for preparations of bacteria stained blue, which stains the nuclei an intense red, the fibrillar substance of the connective tissue pink, and the protoplasmic substance a more or less yellow, so that a threefold staining results. Hæmatoxylin is best used in the following solution:

Hæmatoxylin	2 parts
Alcohol	100 "
Aq. destil	100 "
Glycerine	100 "
Alum. sulph	2 "

This hæmatoxylin stains micrococci, many bacilli, and at the same time the zoöglœa masses, with the intercellular substance. The staining of the bacteria is paler than that produced by the blue or violet basic aniline-dyes, which, on the other hand, do not stain the zoöglœa masses. In order to obtain the threefold staining after the bacteria are stained red by fuchsin, it is better to stain the nuclei with hæmatoxylin, and then afterward stain the protoplasm with a saturated solution of picric acid or eosin. The rose-red eosin can be added to the above solution of hæmatoxylin in the proportion of one half per cent. (To retain the yellow color of the picric acid, picric-alcohol and damar resin must be used.)

OTHER REAGENTS AND APPARATUS.

Other solutions are often required:

(*a*)	Iodine	1 part
	Potas. iodid	2 parts
	Aq. destil	100 "

* Cf. die citirten Handbücher, besonders Friedländer.

(*b*) In the use of the basic aniline-dyes it is seldom necessary to extract the fat. If it is desirable to do this, the sections are first washed thoroughly in absolute alcohol (three to ten minutes), then are transferred to a watch-glass containing ether and chloroform for a few minutes; after this they are again placed in alcohol, cleared up in acetic acid (for the solution of the coagulated albumen), and are then examined immediately or after previous staining.

(*c*) Nitric acid, diluted in the proportion of one part of the officinal acid to three or four parts of water, is sometimes used.

(*d*) For the removal of the lime salts, according to von Ebner, the following solution may be used, but must be often renewed:

Ac. muriat	5 parts
Alcohol	100 "
Aq. destil	20 "
Sodii chlorid	5 "

(*e*) Acetic acid is employed in from one half to one per cent solution for obtaining the maximal decolorization and for the examination of unstained bacteria.

(*f*) Chromic acid is used in a one half per cent solution, or as Müller's fluid:

Potas. bichrom	2 parts
Sodii sulphat	1 part
Aq. destil	100 parts

(*g*) The hydrates of potassium or sodium in one to three per cent solutions, or a solution made by adding one to two drops of the thirty-three per cent solution (so much used in histology) to a watch-glass of water, may be employed for rendering the unstained bacteria visible.

(*h*) Glycerine and alcohol are always to be used only in pure form completely free from acid.

(*i*) The distilled water which is used in bacteriological work should always be sterilized by boiling for an hour over a flame or in a sterilization apparatus, since the ordinary distilled water always contains bacteria and their germs. All the solutions used in bacteriology should be made with sterilized distilled water, and all solutions and reagents must be tested for the possible presence of bacteria. For the preservation of the solutions, flasks with ground stoppers are necessary, and for daily use small glass bottles with hollow-ground stoppers, having above a rubber cap and below a capillary tube, are very convenient. These capillary pipettes serve to draw up many or few drops as is desired.

For the preservation of the bacteria preparations, glycerine can be used only with the brown dyes, since it more or less rapidly extracts the other aniline colors. The glycerine gelatin of Klebs can be used for the brown colors.

The saturated solution of potas. acetat. (1–2) can be often advantageously used for the preservation of the stained and unstained bacteria; but the most universally valuable material for mounting is Canada balsam, which is used most conveniently from a tube (*the artist's ordinary paint-tube*) after having been dissolved.

For diluting Canada balsam, turpentine or xylol must be used, because chloroform extracts the basic aniline-dyes. For the same reason, the precaution should be taken not to warm the balsam. The much-loved oil of cloves should not be employed for clearing up on account of the same objection; but in place of it oil of turpentine, cedar, or bergamot must be used.

In the way of apparatus the following articles are necessary: good slides and cover-glasses; watch-glasses; ordinary porcelain dishes, and those with plain ground bottoms; crystallization-glasses of different sizes, preferably two sizes, of which the greater can be used at the same time as a cover for the smaller; beakers of different sizes; small, square wire baskets for holding test-tubes; test-tubes; wash-bottles; graduates and pipettes; a plate of black glass or porcelain to place under white or unstained objects; a plate of white glass or porcelain to place under stained objects; glass tubes, some drawn out to a capillary-point; glass rods, some having from 3 to 5 cm. of platinum wire of different sizes melted into one end (to be used straight or bent into a loop); spider-forceps; and many slide-pincettes. On one of these latter instruments the ends should be bent out somewhat, and small pieces of flat cork fastened to them for taking hold of cover-glasses. The slide pincettes can be improvised by placing over ordinary pincettes the narrowest possible ring of cork.

Of metal instruments, it is necessary to have some teasing-needles, scissors, knives, and a wide spatula of nickel, steel, or platinum, on which the sections can be spread out in transferring them from one fluid to another.

New slides and cover-glasses are first cleaned with warm water and dried with a linen cloth. But this is not sufficient, and they must then be carefully rubbed with a cloth which has been dipped in spirit. For the preparation of the flat hanging-drop it is often necessary to allow the apparently clean cover-glass to lie some hours in absolute alcohol; to remove the remains of the alcohol by ether, and then, finally, to dry by evaporation. Slides and cover-glasses which

have been used are laid in concentrated muriatic, nitric, or sulphuric acid, and, after cleaning and removal of the acid, are treated with water until all acid reaction has disappeared, and are then further treated as the new.

Cover-glass Preparations.

After it was observed that the morphological elements in the blood, dried in a thin layer, were not materially altered by drying, Koch * first employed these casual observations methodically in bacteria investigation. He spread out upon a cover-glass, in a very thin layer, a drop of fluid containing bacteria, so that the individual elements were brought very nearly on the same plane. This thin layer was then fixed by simply drying in the air. In order to eliminate the slight alteration produced by this, it is necessary afterward to cause again a swelling up of the bacteria. If the layer which has been dried in the air remains too long in the water or glycerine used for this purpose, it is entirely dissolved, instead of only partially swelling.

If the cover-glass with the dried layer is laid in absolute alcohol, or a one half per cent solution of chromic acid, the layer is rendered insoluble in water and glycerine, and no longer swells up. But if the layer which has been made insoluble is put into potassium acetate, it swells sufficiently without being entirely dissolved, and all the forms seem to be in a natural condition. The solutions of the aniline-dye have the same action and cause the same swelling without removing the layer, and at the same time stain the bacteria.

* "Verfahren zur Untersuchung," etc. "Beiträge zur Biologie der Pflanzen," 1877, II, 3. Heft, S. 399.

In the use of this method in the investigation of the blood, Ehrlich * found that the rapid drying prevented coagulation of the cell-albumen, and retained the natural staining capacity of the elements. Only the hæmoglobin was extracted by aqueous and glycerine solutions of the dyes. But if the preparations were kept for a few hours at a temperature of 115°–125° C., the elements of the blood, without any important alteration and without the appearance of artificial products, retained their elective affinities for dyes. In following up these observations, Koch † discovered that, in place of the fixation by alcohol, the application of heat for only a few minutes answered the same purpose.

A drop of the fluid containing bacteria, either undiluted or after the addition of a drop of distilled water (according to the amount of its morphological elements), is spread out in a thin layer upon the cover-glass, by means of a pointed scalpel or platinum wire, and the excess of fluid soaked up with filter-paper; or a drop is placed upon one cover-glass and a second is applied to this, which, through its pressure, spreads out the drop in an even layer. If, then, the two cover-glasses are drawn apart with pincettes, we have two similar preparations. The cover-glasses, protected from dust, are allowed to remain until completely dry, or they can be dried in a dry-oven somewhat more rapidly. The drying can also be hastened by holding the cover-glass with the prepared side upward high above the gas-flame, and moving it to and fro to prevent the direct action of the flame.

Upon the dried preparation a drop of the staining

* "Zeitschrift f. klin. Med.," Bd. I, S. 553.

† "Zur Untersuchung von pathogenen Organismen." "Mittheilungen aus dem kaiserlichen Gesundheitsamte," 1881, Bd. I, S. 1.

solution can then be placed to stain the elements, but only in case the fluid is free from albumen and the staining follows quickly, since, by the prolonged action of the staining solution, the layer is completely loosened. If the dried layer consists of an albuminous substance, such as blood, tissue-fluids, or sputa, on the addition of the staining solution, precipitation occurs.

On this account it is especially necessary that the preparation, after drying in the air, should be more securely fixed by heating. For this purpose the cover-glass may be placed in a drying-box or upon a copper plate. The copper plate is laid upon a tripod, and one end is heated by a gas-flame, so that the different portions, at different distances from the flame, have varying degrees of temperature. A few minutes' exposure to a temperature of 125° C., or ten to twenty minutes at 110° C., is sufficient to thoroughly dry bacteria preparations. This may be done more conveniently, and in some cases also more certainly, according to Koch-Loeffler, if the cover-glasses with the dried layer are drawn rather rapidly three times through a gas- or spirit-flame.

The reason for heating in exactly this manner, according to Koch,* is this: because in preparations which have not been heated the above-described precipitation occurs, while in preparations which have been passed through the flame only once or twice, the fixation of the elements, especially in those containing much albumen, is not sufficient for all cases. In those passed through the flame three times, while the forms themselves are not materially altered, the capacity for staining is retained, and the albuminoid material has become so insoluble that precipitation no longer

* "Mittheilungen," 1884, Bd. II, S. 7.

takes place. Passing the preparations through the flame a yet greater number of times destroys the susceptibility of the bacteria to the staining fluid.

The want of success in making preparations, which many beginners experience, seems to be especially due to the fact that the preparations are generally heated before they have been completely dried in the air. If the preparation still contains any water, coagulation of the albuminoid material occurs when heated, while in those completely free from water this does not happen, and the albumen is rendered homogeneous by heating.

The preparations, dried in the air and then drawn three times through the flame, are now stained. The cover-glasses, with the prepared side upward, are laid on a piece of filter-paper, and, by means of a glass rod, a cap pipette, or the glass stopper with the capillary-tube, a few drops of a staining solution are placed upon the preparation. The staining fluid should remain about twenty minutes, or until it is seen, by an inclination of the cover-glass, that the preparation has already taken up the color. If the action of the staining solution ought to be prolonged, then it should not be placed drop by drop on the cover-glass, because, in drying, the staining solution forms a ring of color at the edge which it is difficult to remove. In this case a sufficient quantity of the staining solution is placed in a watch-glass or crystallization-glass, and the cover-glass is then taken, with the prepared side downward, between the thumb and index or middle finger, and allowed to fall flat upon the surface of the staining solution, so that it swims with the prepared side upon the surface of the fluid. To prevent evaporation, the dish is covered with a glass plate.

For the removal of the excess of coloring-matter, a stream from a wash-bottle is thrown obliquely from above upon the cover-glass, taking care not to strike the surface of the preparation directly; or the cover-glass, held in pincettes, is moved to and fro in a beaker filled with distilled water; or the excess of the fluid may be soaked up with filter-paper, a few drops of water added to be soaked up anew, and so on until none of the coloring-matter is given up to the filter-paper. Then the cover-glass preparation is examined in a drop of distilled water.

The upper side of the cover-glass is freed from every particle of water by soaking it up with filter-paper, because on it must be placed a drop of oil for the homogeneous immersion-lens.

If the cover-glass preparations are to be preserved, the oil is removed with filter-paper and chloroform, the water by careful warming or evaporation (protected from dust), and the dried preparation is directly imbedded in Canada balsam.

Different dyes are used for each variety of bacteria, since some stain only the bacteria; others at the same time the fine gelatinous sheath; others the capsule. On this account the corresponding pictures in all the methods of staining are not absolutely similar; so that it ought to be self-evident that always, in comparison, only preparations should be used which have been treated in exactly the same manner. These considerations must guide one in the choice of the staining solution. We must therefore distinguish between staining for a special purpose—i. e., for the establishment or employment of coloring methods which have been described or proved to be best in particular cases—and the investigation staining used especially to prove the presence of bacteria.

Since in cover-glass preparations almost all bacteria can be stained by watery solutions of the basic aniline-dyes, saturated watery solutions or the equally valuable alcoholic solutions are first employed. The saturated watery solutions have for this testing an advantage, because all basic aniline-dyes are known to be applicable; so, with a few preparations, the different colors can be tried.

If no bacteria come to view in this way, notwithstanding their supposed presence, then aniline-water, with methyl-violet or fuchsin, is used, or the stronger alkaline solution of methyl-blue. The trial examination as to the presence of bacteria resolves itself, in the larger number of cases, into the following procedure:

1. Drying in a thin layer.
2. Fixation by passing the cover-glass three times through the flame.
3. Staining by placing a few drops of a watery or dilute alcoholic solution of a basic aniline-dye upon the preparation.
4. Removal of the excess of the coloring-matter by washing or soaking up with filter-paper.
5. Examination in a drop of distilled water.

For the isolated staining of bacteria in cover-glass preparations they can be laid for about one minute in a half-saturated solution of potassium carbonate; or, if they are stained in aniline-water-gentian-violet, the remaining elements can be decolorized according to the method of Gram.* The stained cover-glass preparations are for this purpose laid for about one minute in the solution of potassium iodide (*vide* page 51), and then placed in absolute alcohol until

* "Ueber die isolirte Färbung der Schizomyceten." "Fortschritte der Medizin," II, 1884, No. 6.

they appear decolorized. The alcohol is soaked out and the preparation examined in water.

For double staining the cover-glass preparations, after being decolorized according to the Gram method, they can be taken from the alcohol and placed in a weak watery solution of vesuvin. Then the bacteria remain blue, often almost blue-black, while the nuclei are stained brown. The preparations stained red or blue can also afterward be stained with carmine or hæmatoxylin; yet this double staining has much less value in the cover-glass preparations than in sections.

EXAMINATION FOR TUBERCLE BACILLI IN SPUTUM.

These preparations can be stained according to the Gram method; but by this both the tubercle bacilli and other bacteria are stained blue in contrast with the brown nuclei. For the differential diagnosis this is not sufficient, and for this purpose the principle established by Koch must be exclusively observed —i. e., that the tubercle bacilli should be stained in a different color from other bacteria and the nuclei. Koch succeeded in doing this, in preparations stained for twenty-four hours in a weak alkaline solution of methyl-blue, and then placed for a short time in a watery solution of vesuvin. In this way the tubercle bacilli (and the bacilli of leprosy) are stained blue; all other bacteria and nuclei, brown. After this important principle was discovered, Ehrlich showed that aniline-water was still a better agent for increasing the intensity of the color, and that in the preparations stained with aniline-water colors the tubercle bacilli withstood decolorization by nitric acid, while all other bacteria were decolorized by this mineral acid. But the preparations can not be left so long in

the acid that complete decolorization occurs, because then also many or all of the tubercle bacilli are decolorized. They should remain in the acid until the red (fuchsin) or blue (methyl-violet) hue has changed into a yellow-red or greenish blue. At this stage the preparations are placed in water, and again a red or blue color appears. By the action of the acid the simple acid union (red or blue) is changed into a triple acid (yellow-red or blue-green), and, by the addition of the water, the triple acid union is destroyed and the red or blue hue reappears. The preparations decolorized by the acid are not washed in water, but in 50 or 60 per cent alcohol; then they are stained in a dilute solution of methyl-blue (or vesuvin). After washing away the methyl-blue (or vesuvin) the preparations are examined in water, or, after removal of the water, preserved in Canada balsam.

After this whole procedure, the tubercle bacilli retain their red or blue color, and are easily recognized among the other elements. Aside from this differential diagnostic action of the double staining, the subsequent staining in another color has an advantage by affording an easier examination of the specimens.

Concerning the choice of the material containing bacteria, it is to be noted that the cheesy masses are to be spread out thin with a sterilized scalpel. Nodules of tubercle must be crushed with a scalpel or between two scalpels, and then be pressed flat upon the cover-glass. The tough, yellowish masses from the sputum are used. One of these particles is taken and spread out in a thin layer on the cover-glass, or flattened by pressing one cover-glass upon another, so that, after separating the two cover-glasses with

pincettes, two preparations are obtained. The entire method is, according to Koch (after the adoption of the previously described aniline-water staining of Ehrlich), briefly as follows:

1. Pass the dried cover-glass preparations three times through the flame.

2. Stain with the Weigert-Koch solution of methyl-violet or fuchsin for twelve hours.

3. Treat with dilute nitric acid (1 to 3 or 4) for a few seconds.

4. Wash in a 60 per cent solution of alcohol by a to-and-fro motion.

5. Stain in a dilute solution of vesuvin or methyl-blue.

6. Wash and examine in water or mount in balsam.

This method is the best thus far discovered, and serves as a control in all doubtful cases.

For the differential diagnostic decolorization and subsequent staining of preparations of tubercle bacilli, the following reagents have been used:

1. Other aniline-dyes (vesuvin by Koch).

2. Acids (nitric by Ehrlich, hydrochloric by Orth, acetic by Petri).

3. Acid alcohol (weak nitric by Rindfleisch, and hydrochloric by Orth).

B. Fraenkel combined these three variations by preparing an acid-alcoholic solution of methyl-blue and of vesuvin.

a. For blue:

Alcohol	50 parts.
Water	30 "
Nitric acid	20 "

Methyl-blue to saturation.

To be filtered.

b. For brown:

Alcohol	70 parts.
Nitric acid	30 "
Vesuvin to saturation.	

To be filtered.

For the use of these solutions the following method of Fraenkel is recommended: About 5 c. cm. of aniline-water are heated in a test-tube to boiling and poured out in a watch-glass; to this hot aniline-water a concentrated alcoholic solution of fuchsin or methyl-violet is added, drop by drop, until the solution of the dye assumes a cloudy appearance. Upon this warm solution the cover-glass preparations are allowed to swim, and even in two or three minutes most of the tubercle bacilli are stained; but, as a precaution, they should be left five or ten minutes. From this staining solution, the preparations stained red or blue are passed into the blue or brown acid-alcohol solution. After remaining one or two minutes in the latter, the preparations seem colored and are washed in water or 50 per cent alcohol, to which one half per cent acetic acid has been added, and are then examined in water.

Those who prefer the hydrochloric acid of Orth can employ the following method of Kaatzer: stain as before, and then decolorize with a mixture of—

Alcohol (90 per cent)	100 c. cm.
Water	20 c. cm.
Concentrated hydrochlor. acid	20 gtt.

wash with 90 per cent alcohol to remove the acid, and then stain with a concentrated watery solution of methyl-blue or vesuvin.

In sputum, according to Celli and Guarnieri, sometimes very fine fat-crystals are found, which react to the staining almost as the tubercle bacilli, "pseudo-

bacilli," but which, on careful examination, are not to be confounded with them, on account of their varying size and because they are dissolved by ether and chloroform.

The published modifications of the Ehrlich method, founded on the Koch principle, are so numerous, but without anything having been added, that I must on this account simply refer to some of the comprehensive descriptions,* and content myself with giving the underlying method of Koch (which is now best employed according to Ehrlich-Weigert-Koch), and two practical modifications of it.

In these methods the bacilli of leprosy react as the tubercle bacilli, from which, moreover, they are morphologically not easy to differentiate. The differential diagnosis by staining is founded on this fact: that the bacilli of leprosy do not stain so deeply as the tubercle bacilli, and that, in respect to the ease with which they give up the dye, they stand between these and most other forms of bacteria. According to Baumgarten,† the dried cover-glass preparations are allowed to float for six or seven minutes in a dilute alcoholic solution of fuchsin (5 or 6 drops of a concentrated alcoholic solution in a watch-glass of distilled water), decolorized fifteen seconds in acid alcohol (1 part nitric acid to 10 parts alcohol), washed in distilled water, afterward stained in a watery solution of methyl-blue, washed again, and examined in water.

* Kaatzer, "Die Technik der Sputumuntersuchung auf Tuberkel-Bacillen," 1884. B. Fraenkel, "Ueber die Färbung des Koch'schen Bacillus," "Berl. klin. Wochenschrift," 1884, No. 13. Baumgarten, "Beiträge zur Darstellungsmethode der Tuberkel-Bacillen," "Zeitschrift für wissenschaftliche Mikroskopie," I, 1884, S. 51.

† "Ueber Untersuchungsmethoden zur Unterscheidung von Lepra- und Tuberkel-Bacillen," ibid., S. 367.

The bacilli of leprosy seem then as red rods upon a blue ground, while the tubercle bacilli during this time, by this treatment, have as yet taken up no color.

The reaction of the tubercle bacilli, both in the method of staining discovered by Koch with its different modifications, and in the method of Baumgarten (the recognition of these bacteria in unstained conditions), seems at first to separate these bacilli qualitatively from all other bacteria. Further studies, however, have shown that these differences are not qualitative, but are especially quantitative. The tubercle bacilli are stained with the greatest difficulty; but they retain the color persistently. It has been shown since by Lichtheim,* and especially by the work of Baumgarten, previously cited, that the tubercle bacilli in dried cover-glass preparations are stained in about one hour, both in dilute alcoholic and strong aqueous solutions of methyl-violet, gentian-violet, and fuchsin, or by simultaneous warming, in about five minutes. (For sections the time required is about twelve hours, or about ten minutes with elevation of temperature.)

These stainings withstand the decolorization by acids for some time, although after a longer action of the same the tubercle bacilli are also decolorized. The tubercle bacilli remain unchanged after treatment with carbonate of potassium, as do other bacteria. If the stained cover-glass preparations are laid for decolorization for about one minute (sections, five minutes) in alcohol, and afterward for five minutes (sections, fifteen to twenty minutes) in a concentrated aqueous solution of vesuvin or methyl-blue, a double staining is obtained. Therefore, neither the addition of the alkali nor the aniline-water is absolutely essen-

* "Zur diagnostischen Verwerthung der Tuberkel-Bacillen." "Fortschritte der Medizin," 1883, S. 1.

tial to the staining; the reaction to acids is not a qualitative differentiation from other bacteria, and the use of the acid is not entirely necessary for obtaining the double staining.

A satisfactory explanation of the theory of staining, which seems to lie so near, has not yet been discovered, on account of the varying quantitative reaction of the tubercle bacilli. The possibility of a better understanding is offered by the following fact: The addition of an alkali, as well as the feebly alkaline aniline-oil, renders the staining easier. Other aromatic bodies and ammonia act in a similar manner. The addition of an acid to the aniline-water does not arrest its action, so that probably the favorable action of carbolic acid is reduced to the aromatic element, and is effectual in a similar manner in spite of the acid reaction.

In addition, Gibbs* showed that in the simultaneous action of two aniline-dyes the bacilli are stained differently from the other elements, while by another method these are stained in both of the dyes. A solution of 3 c. cm. of aniline-oil in 15 c. cm. of spirits is added slowly to 2 grammes of fuchsin and 1 gramme of methyl-blue, and, after the solution of the dyes, 15 c. cm. of water are added. This solution is warmed, the cover-glass preparation is laid upon it for five minutes, and then washed in spirits till color is no longer given up. The bacilli then appear red upon a blue ground. This method is, unfortunately, not sufficiently reliable.

EXAMINATION OF BLOOD FOR BACTERIA.

The examination of blood for bacteria offers very great difficulty, because in the normal blood within

* "Lancet," 1883, p. 771.

the vessels, and in the normal disintegration of the healthy blood, granular elements are present, or are formed, which, under certain pathological conditions, in anæmic states and in fever, are increased in number, and can be easily mistaken for micrococci. They have already been often confounded with micrococci, and are almost daily mistaken for them—e. g., the renowned syphilitic corpuscle, and the so-called organisms of the venom of serpents. Here belongs also much of what has been spoken of as the development of bacteria from nitrogen molecules, from microzymen, or from the anamorphosis of protoplasm. An exact study of these granules of the blood is on this account an indispensable desideratum in bacteria investigation.

These granules form, further, a constituent part of the cellular elements of the blood, and on this account again are of interest in ætiology, because there are parasites which are similar to the amœboid cells —e. g., those monads found by Lewis in the blood of rats, by Koch in the blood of marmots. The elements of the blood, which directly or through their granules may be confounded with micro-organisms (with the exception of the red blood-corpuscles, and the products of their disintegration), are divided, according to Ehrlich,* into—

1. Lymphoid elements.

(*a*) Small lymph-cells. (*b*) Large lymph cells.

2. Myeloid cells (eosinophile).

3. Undetermined (spleen and [or] marrow).

(*a*) Large mononuclear cells. (*b*) Transitional forms. (*c*) Polynuclear.

* Cfr. the work of Ehrlich, Westphal, Schwarze, and Spilling, "Ueber Blutuntersuchungen bei Leukämie," Dissert., Berlin, 1880; and Einhorn, "Ueber das Verhalten der Lymphocyten zu den weissen Blutkörperchen," Dissert., Berlin, 1884.

The small lymphoid elements are somewhat smaller than the red blood-corpuscles, possess a very large nucleus, so that there is very little or no protoplasm to be seen. The large lymph-elements are a further development of the first, and are only to be differentiated from them in this, that they possess around the large nuclei a distinct border of protoplasm. The myeloid elements are large, round cells, with large, oblong nuclei. The mononuclear cells are about three times the size of the red blood-corpuscles, and possess round or oval nuclei of large size, and a considerable mass of protoplasm. The mononuclear transitional forms are to be differentiated from these cells only in this, that the nuclei are no longer round or oval, but have become indented. The polynuclear elements are somewhat smaller, but still are always larger than the red blood-corpuscles, and their nuclei show, as a further differentiation, a polymorphous form. These are the true white blood-corpuscles. The granular elements, or granules, which are present in the cells, and which become free in the destruction of the same, are divided with respect to their reaction to aniline-dyes.

The α, or eosinophile granule, is coarsely spherical, strongly refracting, and can be stained in all the acid aniline-dyes. It is present in the myeloid elements, seldom in the normal blood, and its number is greatly increased in leucæmic processes.

The β, or amphophile granule, is found especially in the marrow, very often in the leucocytes in the blood of rabbits and guinea-pigs, and can be stained by acid and basic aniline-dyes.

The γ, or basophile plasma cell granule, can be stained, like the bacteria, by the basic aniline-dyes. These granules are coarse, slightly refracting, almost

completely wanting in normal human blood, increased in leucæmic processes, and are present normally in the blood of the lower animals, especially the white rat.

The δ, or basophile granule, is fine, and can be stained in basic aniline-dyes, and forms a constituent part of the large mononuclear elements.

The ϵ, or neutrophile granule, is very fine, and fills the polynuclear elements of the human blood quite thickly, is present sparsely in the transitional forms, and very seldom in the mononuclear elements. It can be stained by the neutral dyes.

Without recourse to staining, these granules, as a whole, and also the products of the disintegration of the red blood-corpuscles, may be confounded with micrococci. By systematic staining with the aniline-dyes, the α, β, and ϵ granules can be excluded. An error is then possible only with the γ and δ granules, because these are stained in the basic aniline-dyes, the same as the bacteria. These last, on account of their fine grain, can, with comparative ease, be differentiated from micrococci, and have not, as yet, been confounded with them. The plasma cell granules, on account of their medium size, come so near to the known forms of cocci, that not only the individual free granules in the blood have been considered as cocci, but even the same so-called plasma cells in the tissues have been described as colonies of cocci. They can, on purely morphological grounds, be differentiated from them in this way, viz., that they do not all have the symmetrical appearance of the cocci, but present the greatest differences in the size of the granules.

If it is desired to examine the blood for bacteria, a small drop is rapidly spread out in a thin layer, dried, fixed, and then passed three times through the

flame, and then stained in the ordinary manner. In such preparations the bacteria are sufficiently stained, but not the granules.

The small drop of blood, at most the size of a pin-head, must be taken with the greatest care. In case of a hæmorrhage, a small drop is taken with a sterilized platinum wire, or, for obtaining the blood, the skin may be pricked with a previously sterilized needle, the end of the finger being the best spot. The skin is first cleansed with a brush and soap, and then washed with a solution of corrosive sublimate, 1 to 1,000. The sublimate is removed with alcohol, the alcohol with ether, and the last allowed to evaporate. The first drop of blood which wells up is removed with a sterilized platinum wire, and the following drop used, a cover-glass being lightly pressed upon it with the pincettes, without coming in contact with the surrounding skin. Upon this first cover-glass a second is laid, which, by pressure, spreads out the blood in a thin layer, in which the elements are not materially altered. The two cover-glasses are then drawn apart by pincettes, so that two cover-glass preparations are obtained. Some of the cover-glasses are used as above in the examination for bacteria, after the layer has been dried in the air, and heated only for a short time ; but others are heated for one hour at a temperature of 120° C., and then treated with a basic aniline-dye, in order to study more exactly the basophile granules.

Other preparations for the determination of the eosinophile elements are treated with the acid aniline-dyes after heating a short time, and also after an hour's heating. A mixture is made of the yellow, red, and black dye-stuffs of the strongest staining power, each of which alone stains all of the acid-

forming elements, and both the simultaneous action and the elective staining are valuable, since the three eosinophile elements are stained at the same time in different colors. One part of a saturated glycerine solution of aurantia is diluted with two parts of glycerine; then aniline-black (sulphate of indolin) and eosin are added in excess, and, by long shaking, dissolved to saturation. This saturated glycerine solution stains all the parts containing hæmoglobin intense orange, the nuclei gray-black to black, and the eosinophile granules red to red-black.

For staining the neutrophile granules the neutral dyes are used, which are formed by the union of the basic and acid dyes—e. g., if acid-fuchsin and orange (G) are mixed with the basic methyl-green. According to Ehrlich, 125 c. cm. of a saturated aqueous solution of orange are mixed with 125 c. cm. of a saturated solution of acid-fuchsin in 20 per cent alcohol; 75 c. cm. of absolute alcohol are then added, and afterward 125 c. cm. of a saturated aqueous solution of methyl-green with shaking.

The solution remains standing for some time, and both a precipitation occurs and a film forms on the surface. In order to obtain the solution quite clear, a pipette is introduced into the middle of the solution and a quantity of the clear fluid is withdrawn. The pipette as well as the vessel must be absolutely dry, or otherwise a further cloudiness appears. By this mixture the hæmoglobin is stained yellow to orange, the nuclei green, neutrophile granules violet, and the eosinophile dark gray with a tinge of blue.

For the demonstration of the cells in the blood the following mixture is used: *

* Ehrlich, "Deutsche med. Wochenschrift," 1883, No. 46.

Water	100 c. cm.
Glycerine	100 "
Absolute alcohol	100 "
Hæmatoxylin	1–2 grm.
Eosin	1 "
Glacial acetic acid	10 c. cm.
Alum to saturation.	

The red blood-corpuscles show an intense red color, the nuclei of the lymphoid and polynuclear cells are stained intensely blue, and the nuclei of the mononuclear cells bluish green. The protoplasm of the large lymphoid and polynuclear cells is reddish, that of the mononuclear cells dark green.

The importance of this method of examination for micro-organisms in the blood has been lately pointed out by Koch;* but it has not as yet received necessary attention. The descriptions given here are all the more needful because our good text-books on histological *technique* do not sufficiently treat this subject, for the reason that the original works are so difficult of access.

Methods of Staining the Flagella.

The flagella (Fig. 4; 7, 9, 14, 16), which become visible in the hanging-drop at one or both extremities of the bacteria by forming an eddy, can be best stained in dried cover-glass preparations, according to Koch,† by the addition of a concentrated aqueous solution of campechianum. The flagella are stained brown, but the staining in this manner is not durable. On this account the stained preparations are laid for some time in a 5 per cent solution of chromic acid or

* "Mittheilungen," Bd. I, 1881, S. 7.

† "Verfahren zur Untersuchung, etc." "Beiträge zur Biologie der Pflanzen," 1877, Bd. II, 3. Heft, S. 419.

in Müller's fluid. Then there is formed an insoluble brownish black union of the extract of hæmatoxylin with the chromic acid. After washing, these preparations can be directly preserved in glycerine, or, after drying, in Canada balsam.

Methods of Staining Spores.

The spores of bacteria were first observed and described, but not rightly understood, by Perty.* Then Pasteur† made a sharp distinction between the biology of an organism and its spore without quite solving the question morphologically. Cohn‡ was the first to describe biologically and morphologically the formation and germination of spores. Further peculiarities were observed by Koch, Brefeld, Buchner, and especially Prazmowski,# who clearly described the different forms of the germination of spores (Fig. 4; 18 and 19). By his observations this process of fructification derived a heightened significance.

The observation of the spores in the unstained condition finally followed (Fig. 4; 5 b, 8, 9, 10, 12). They appear, especially in the hanging-drop, as strongly refracting round or oval bodies either within the less refractive bacteria, or free near these. Sometimes they are situated near the middle, sometimes at the end. The cells in which they appear are sometimes unaltered, sometimes peculiarly swollen. Since con-

* "Zur Kenntniss kleinster Lebensformen," 1852. Taf. XV, Fig. 26, and following.

† "Études sur la maladie des vers à soie," 1870, I, S. 228.

‡ "Beiträge zur Biologie der Pflanzen," 1876, Bd. II, 2. Heft, S. 263.

"Untersuchungen über die Entwicklungsgeschichte und Fermentwirkung einiger Bakterien-Arten," 1880, und "Ueber den genetischen Zusammenhang der Milzbrand und Heubakterien." "Biolog. Centralblatt," 1884, No. 13.

densed bacteria-protoplasm, according to Prazmowski, strongly refracts light, it is to be concluded that a body still more strongly light-refracting is to be regarded as a spore.

Here belong, together with the foregoing morphological changes which regularly appear under certain biological conditions in spores, their great resistance to chemical agents, and especially to high temperature, and the fact that in the use of watery or dilute alcoholic solutions they are not stained, but appear as unstained refracting spaces within the stained bacteria.

An accidental observation showed how the spores could also be brought to view stained. Koch * saw, in staining the tubercle bacilli with aniline-water-methyl-blue, that the spores of a species of large bacteria were stained blue at the same time, while the bacteria themselves were stained brown by the subsequent treatment. Gaffky was not able to stain the spores of other bacteria in the same manner. On the contrary, Neisser succeeded in staining the spores red and the bacilli blue when he used warm aniline-water-fuchsin and subsequently stained with methyl-blue. Bienstock † also used this staining. Further, Buchner ‡ discovered a means for the isolated staining of spores. Because the staining of the living bacteria was not successful, but those killed by drying and heating were readily colored, Buchner thought that the reason the spores were not stained was because of the greater resistance of the spore-membrane. He endeavored, therefore, to destroy the membrane of the spores of the bacillus subtilis, and

* "Mittheilungen," 1884, Bd. II, Tafel V, Fig. 23.

† "Zeitschrift für klin. Med.," 1884, S. 1.

‡ "Ueber das Verhalten der Spaltpilzsporen zu den Anilinfarben." "Aerztliches Intelligenzblatt," 1884, No. 33, S. 370.

thus make them accessible to the staining fluid. In this manner he succeeded in staining spores in dried cover-glass preparations which had been heated from one half to one hour at a temperature of 210° C. in a dry-oven, or one hour in a steam-kettle at 120° C. A successful result was also obtained when the preparations were dipped in concentrated English sulphuric acid for fifteen seconds and afterward carefully washed, or when they were subjected for a longer time to a concentrated solution of caustic soda. In preparations thus treated, especially in the use of methyl-blue, the spores alone are stained, while the bacteria themselves no longer take up color.

Even before the publication of the brief article of Buchner, I had endeavored to bring the spores into view by both isolated and double staining. Although these investigations are not yet in all directions concluded, still I shall now describe the general results, because this part of the *technique* is as yet little known and used.

If bacteria are examined in the hanging-drop shortly before the formation of spores, in many of them refracting bodies are found which have not yet a size equal to that of spores. When the preparations, dried and fixed by passing them through the flame three times, are stained with an aqueous or dilute alcoholic staining solution, the bacteria in this stage are found to be stained, not so equally as before, but some parts are more deeply colored. This condensed protoplasm takes the dye more readily than the protoplasm not thus concentrated. (I had previously noticed intimations of this in the involution forms of the bacillus cyanogenus.) Then follows the stage in which the refracting corpuscles become much more equal in size, but they still stain well; and finally

a stage in which similar refracting corpuscles are present in the unstained preparations, but these no longer take up the color. Now the spores have first obtained an insusceptibility to the dye through the formation of a membrane difficult of penetration, which prevents the absorption of the coloring-matter.

In the cover-glass preparations, which have been passed through the flame three times, the bacteria and nuclei are stained well. If they are passed through the flame more times, say six, the bacteria are stained successively worse, but the nuclei still absorb the dye well, as does also the condensed protoplasm of the bacteria which has not yet formed spores. At this stage, besides the nuclei, granular elements can often be seen which may easily impress one as belonging to the badly stained bacteria. If the preparations are passed through the flame still more times, say ten, then both the nuclei and the condensed protoplasm lose their susceptibility to the dye, and the spores gain it.

In the case of some of the bacteria of putrefaction, it is sufficient to pass the preparation through the flame only seven times, but in others ten times are required. (In the dry-oven a similar stage is reached in from fifteen to thirty minutes at 180° to 200° C.) The spores then take up aqueous solutions of red, violet, blue, brown, and green basic aniline-dyes.

This isolated staining for the proof of the resistance of the spores, as Buchner intended, is perhaps sometimes to be used; but, since in this way nothing is learned concerning the relation of the spores to the bacteria, it is better to use a double staining. The procedure is almost the same, quantitatively increased, as that for staining the tubercle bacilli.

Either the preparations, passed through the flame three times, are stained with a strong alkaline solution from twelve to twenty-four hours (less advantageously by warming for one hour), and afterward stained with vesuvin, or the aniline-water-dye solutions are used, of which that of Neisser, previously described, has proved to be the most convenient and satisfactory. Stain in hot aniline-water fuchsin, decolorize with nitric acid, then stain with methyl-blue.

Some spores are stained, moreover, by saturated aqueous or dilute alcoholic solutions, if they are at the same time heated. The difference of spores, in respect to their susceptibility to dyes, seems to be scarcely less than that of the bacteria themselves.

Preparation of Sections.

Pieces of fresh organ can be cut with the freezing microtome. The sections are placed in a one half per cent solution of sodium chloridum, and in part examined fresh, in part stained. For the latter purpose, the section, according to Weigert,* is spread out upon a section-lifter in the salt solution with the help of a needle, is then taken out, and the excess of the fluid removed with filter-paper. Then the section is placed in absolute alcohol, introducing it slowly, where it remains at least until the disappearance of all the air-bubbles arising from the thawing of the frozen specimen. Then the section is placed in a staining solution, for which purpose vesuvin is most recommended, because for staining in the other dyes the sections must remain longer in alcohol. According to Friedländer,† the section can also be taken out

* Virchow's "Archiv für pathologische Anatomie," Bd. LXXXIV, 1881, S. 290.

† "Mikroskopische Technik," 2. Aufl., S. 119.

of the salt solution and placed directly in the brown staining fluid, then for a short time in alcohol, and finally in glycerine, or oil of cloves and Canada balsam.

For the study of bacteria, it seems to me that the preparation of fresh sections is entirely superfluous work. In the same time in which available sections for this purpose can be made, a dozen dried cover-glass preparations of the tissue-juice can be obtained, which furnish better information as to the presence and import of bacteria.

For the exact study of the presence and distribution of bacteria in the tissues, it is necessary to harden the tissues thoroughly, and from the hardened specimens to prepare with the microtome a series of delicate sections. These sections are partly examined unstained and partly after staining. Since both the staining itself, as well as the maximal decolorization, is dependent upon the condition of the preparation produced by hardening, it is of the first importance for bacteria specimens to use absolute alcohol as a hardening agent. The susceptibility to dyes, of tissues hardened by other agents, as chromic-acid salts, is inconstant; but possibly these hardening agents are for other parasitic micro-organisms as valuable as alcohol. (Cf. page 68.)

The susceptibility of the albuminates to dyes depends very much upon the presence of water; and since the albumen coagulated by alcohol retains a certain amount of water, which in the course of a few days is entirely removed by the absolute alcohol, the hardening in alcohol must continue until this condition is constant. For this purpose a small piece of the organ, about the size of a hazel-nut, is allowed to remain at least three days in a large quantity of frequently changed absolute alcohol.

In unstained sections from fresh or hardened tissue, the bacteria are made visible by their resistance to acids and alkalies.* The sections are fully cleared up by 50 per cent acetic acid or 1 to 3 per cent caustic soda or potash. The bacteria withstand the action of these reagents, as was shown by von Recklinghausen (page 38). Old spirit preparations are heated in these solutions to near the boiling-point. In preparations which have thus been made transparent, the bacteria can be sometimes recognized by the characteristic forms of the individual germs, as was found by Baumgarten (page 38) for the tubercle bacillus, and by Friedländer for the typhoid bacillus. In the case of bacteria not distinguishable by the characteristic form of the single individuals, especially in the cocci, their grouping as diplococci, sarcina, torula, or zooglœa is often characteristic. These bodies, of equal size, withstand the action of ether and chloroform, unlike fat-drops, which may be confounded with them. In the vessels the masses of cocci are characterized, according to von Recklinghausen, by the production in the course of their growth of a varicose condition of the vessels.

If we find, in a section of fresh or hardened tissue, masses or chains of small bodies which are of a similar size, and which withstand both the action of alcohol and ether, and the treatment with concentrated acetic acid and the alkalies after warming, these are to be considered, according to Friedländer,† as microorganisms.

More important is the determination, by staining, of the presence of bacteria in sections hardened in al-

* Friedländer's "Zusammenstellung in der mikroskopischen Technik," 2. Aufl., S. 45.

† "Mikroskopische Technik," 2. Aufl., S. 46.

cohol. The necessary duration of the hardening of tissues in alcohol, to produce constant reaction to dyes, diminishes the susceptibility of many elements to the dyes, so that the staining of the hardened sections is, as a rule, a quantitative increase of the methods described for the dried cover-glass preparations. The sections are first overstained, and then, by a second maximal decolorization, reduced to a proper degree of color for the nuclei and bacteria.

The sections which have been cut in alcohol and afterward placed in the same fluid are put into a saturated aqueous or diluted alcoholic solution of the dye, in which they should remain from five to thirty minutes. By warming to 40° or 50° C., the time required can be shortened somewhat, and often also the intensity of the staining be increased. Gentian-violet, according to Weigert, is used very advantageously in a 1 per cent aqueous solution. The sections are stained diffusely in this.

In this method of staining the previously much-used decolorization by acetic acid is omitted, because many bacteria, such as the bacillus of typhoid fever and glanders, give up their color more or less completely in a short time. The sections are placed in distilled water to remove an excess of the color. They are then carefully spread out upon a section-lifter, and with this slowly dipped in absolute alcohol for differentiation of the bacteria and nuclei and for dehydration. They remain for a few minutes in alcohol, which must be absolutely free from acid, then are cleared up in oil of turpentine or cedar, and can be immediately examined in this medium. For preserving, the oil is removed with filter-paper, and they are then mounted in Canada balsam. Since the balsam is soluble in the immersion-oil, it must be suf-

ficiently hard to prevent solution by the shifting of the cover-glass. If it is desired to examine or send away preparations freshly mounted in balsam, it is advisable to fix the cover-glass upon the slide with shellac or gold-size, which are not soluble in the immersion-oil. Many different dyes are also available for use in staining sections.

In the use of the method of staining just described, the typhoid bacillus stains badly; the spirillum of relapsing fever only in brown, and then not well; and the bacillus of leprosy badly in brown, but well in red and blue dyes. For these cases the greatest intensity is obtained by the use of the strong alkaline solution (page 49), which, on this account, according to Löffler, up to the present time has proved the most universally valuable solution for sections.

The sections are placed in this solution for a few minutes, then for a few seconds in ½ to 1 per cent acetic acid, and moved to and fro in order to remove the excess of color from the tissue and to differentiate the bacteria and nuclei; then are dehydrated in alcohol, cleared up in oil of cedar, and preserved in Canada balsam. By this treatment many forms of bacteria, otherwise difficult to stain, are well stained; the tubercle bacillus as well as by other methods, also the bacillus of glanders (which in the aniline-water solutions is decolorized by the acetic acid); the typhoid bacillus and the spirillum of relapsing fever, which had been previously stained only very defectively in any other manner. In the case of most other bacteria, no great difference has been noticed as to the value of this method. The comparative staining by the use of aqueous and alkaline solutions sometimes allows the recognition of pure microscop-

ical differences which are available for the differential diagnosis in morphologically similar forms.

For the isolated staining of bacteria in sections, according to Koch, the sections are brought from the staining solution into a solution of potassium carbonate, which is prepared by the mixture of equal parts of distilled water and a saturated solution of the salt. The sections remain in this solution for about five minutes, and then are transferred with a section-lifter to alcohol, cleared up in oil of cedar, and preserved in balsam. In this method different dyes can be used. If the sections are stained in the aniline-oil-gentian-violet solution, the isolation, according to the Gram method (page 60), is still more beautiful for most bacteria. The sections are taken from the alcohol in which they are placed after cutting, and put from one to three minutes (tubercle bacilli in sections twelve to twenty-four hours) in an aniline-water solution of gentian-violet. Then, either without washing or after gently washing in alcohol, they are transferred to the iodine and potassium iodide solution, in which they remain one to three minutes. In the iodine solution a precipitation occurs, and the sections are stained a blackish purple; they are then placed in alcohol until completely decolorized, cleared up in oil, and preserved in balsam as usual. The bacteria appear dark blue, the nuclei and tissue pale yellow. The capsule-cocci of pneumonia (at least as a rule), and the typhoid bacilli are decolorized like the nuclei.

Double staining can be obtained in sections which have been treated, according to the Gram method, by placing them in an aqueous solution of vesuvin after the decolorization in alcohol. They are then dehydrated in alcohol, cleared up in oil, and mounted in

balsam. The nuclei are stained brown, while the bacteria remain blue.

As already stated, carmine and hæmatoxylin do not stain all bacteria, and even those stained are not as well stained as by the basic aniline-dyes; but they are nuclei-staining agents of the first order. Sections in which the bacteria are stained blue, according to the Koch method, can afterward be placed in a solution of carmine or hæmatoxylin for about ten minutes, in order to stain the nuclei. They are then likewise treated with alcohol, oil, and balsam.

If the sections are diffusely stained in concentrated aqueous or diluted alcoholic solutions of dyes, they are first put into alcohol for the differentiation of the bacteria and nuclei; then, for the removal of the alcohol, for a moment in water, and afterward into a solution of carmine or hæmatoxylin, according as the first staining was with a blue or red aniline-dye.

For these cases, according to Weigert, a 1 per cent solution of gentian-violet and a subsequent staining with picro-carmine is especially recommended. The time required for the ordinary nuclei staining (about ten minutes) must here be somewhat extended, because the carmine must displace the gentian-violet from the nuclei. The time required is from a half-hour to an hour. By a still longer action the carmine is also partially substituted for the aniline-dye in cocci.

These different elective affinities of the bacteria and nuclei for dyes can be used, perhaps, yet more advantageously if the sections are first placed for ten or fifteen minutes in a solution of carmine or hæmatoxylin. In this time a good and durable staining of the nuclei is obtained, while the bacteria are either unstained or badly stained. After washing in water,

the sections are then transferred to a solution of one of the basic aniline-dyes in which the bacteria are stained, while the first dye-stuff is not displaced from the nuclei. They are then treated as usual with alcohol, oil, and balsam. If picro-carmine is used in place of the ordinary carmine, a threefold staining is obtained (page 51).

The so-called plasma-cells in sections can be easily confounded with cocci. They are spherical or spindle-shaped cells with a coarsely granular protoplasm, the granules of which react to the basic aniline-dyes in the same manner as most cocci. The nuclei of these cells are not stained, so that the appearance of colonies of cocci is closely simulated, with which they have often been confounded.

These granules do not show the same resistance to acids and alkalies, and are not of quite such equal size as the cocci. Further, they are especially to be recognized by their position, as they are commonly found on the walls of the vessels. A careful study of these cells is in the highest degree essential, and the ear of the white mouse is especially recommended for this purpose. An exact differentiation is often only to be obtained by the isolated staining of the bacteria, in which the plasma-cell granules remain unstained.

For the simultaneous staining of most cocci and the plasma-cells, the following solution of Ehrlich and Westphal is recommended, which also permits of the differentiation of other bacteria from the nuclei:

Partsch-Grenacher's carmine (carmine pur., prts. 2; aq., 200; alum, 5; boil for fifteen minutes; filter; add carbolic acid, 1 part)..... 100 c. cm.
Glycerine..... 100 c. cm.
Conc. alcoholic sol. dahlia..... 100 c. cm.
Glacial acetic acid..... 20 c. cm.

In this solution the sections remain twenty-four hours, then are transferred for a few minutes to alcohol, afterward treated with oil and balsam. In this solution the nuclei take a red, the plasma-cell granules and bacteria a blue or violet color.

For the demonstration of the capsule cocci of pneumonia, Friedländer now uses:

Conc. alcoholic solution gentian-violet.....	50 c. cm.
Aq. destil.....	100 c. cm.
Ac. acetic.....	10 c. cm.

The sections remain in this solution twenty-four hours, and then are placed for differential decolorization in 1 per cent acetic acid for a few minutes; afterward, as usual, in alcohol, oil, and balsam.

A contingent differential diagnosis is possible microscopically from the fact that by the use of the Gram method these capsule cocci are decolorized, while the remaining cocci all (?) retain the color.

Babes* recommends safranin for the staining of bacteria in sections. The sections are allowed to remain a half-hour in a mixture composed of equal parts of a concentrated aqueous and a concentrated alcoholic solution of the dye. Then they are put for a short time in water, and a few minutes in alcohol; afterward in oil of turpentine and balsam. The bacteria are stained red, and sometimes have almost an isolated staining. This method offers no advantage, since the cocci are well stained, but other bacteria partly not at all and partly much worse than by other methods.

The typhoid bacilli are ordinarily more difficult to stain than most other bacteria, even if the solution is warmed. According to Gaffky,† it is best to allow

* "Archiv f. mikroskopische Anatomie," Bd. XXII, S. 359.

† "Mittheilungen," 1884, Bd. II, S. 378.

the sections to remain from twenty to twenty-four hours in a deep-blue opaque solution, which is prepared fresh each time by the addition of a saturated alcoholic solution of methyl-blue to distilled water. They are then washed in distilled water entirely free from acid, dehydrated in absolute alcohol, cleared up in oil of turpentine, and preserved in balsam. They are also well stained in the alkaline solution of methyl-blue, while by the use of the Gram method they are decolorized.

The bacilli of glanders are decolorized, after staining in an aniline aqueous solution, by water containing acetic acid. However, they are well stained in an alkaline solution of methyl-blue.

The bacilli of leprosy in sections are stained the same as tubercle bacilli. For differential diagnosis Baumgarten recommends the following (page 65): The sections are placed for twelve, or at most fifteen, minutes in a dilute alcoholic solution of fuchsin, then thirty seconds in acid alcohol (nitric acid one part, alcohol ten), to decolorize them; washed in distilled water, dehydrated in alcohol, then treated with oil and balsam. In this time the bacilli of leprosy are well stained, while the tubercle bacilli are not stained at all.

The tubercle bacilli can be brought out if the sections are placed for twelve hours in a weak alkaline, or for one hour in a strong alkaline solution of methyl-blue (page 49), then for a few minutes in a concentrated aqueous solution of vesuvin, and finally in alcohol. They can also be made visible by the Gram method. For the differential diagnosis the sections are stained with the greatest certainty, according to the previously described principle, in the aniline-water-dye solutions of Ehrlich or Weigert-Koch.

The sections remain twelve to twenty-four hours in an aniline-water, methyl-violet, or fuchsin solution, then a few minutes in dilute nitric acid (1 to 3 or 4); are washed in 60 per cent alcohol for a few minutes, stained in a dilute aqueous solution of vesuvin or methyl-blue, washed in 60 per cent alcohol, dehydrated in absolute alcohol, cleared up in oil of cedar, and finally imbedded in Canada balsam.

The vibriones of cholera Asiatica can be stained in sections like most bacilli.

The spirilla of relapsing fever are stained in aqueous and glycerine solutions of vesuvin, but not well in other dyes. On the other hand, they are stained well in strong alkaline solutions of methyl-blue.

A few remarks are still necessary concerning the epiphytic bacteria and the other epiphytic microorganisms similar to them. (Whether the parasitic bacteria are to be classed with the epiphytic has not yet been certainly determined.) The chief difficulty in this investigation is due to the presence of fat. Balzer* washed the scales of the epidermis with ether and alcohol, and examined them after staining with an alcoholic solution of eosin, or without previous staining, in a 40 per cent solution of caustic soda. According to von Sehlen,† the hairs, after the removal of the fat, are placed for a short time in alcohol, and then in a dilute aniline-oil solution of fuchsin. They are afterward washed in acid alcohol (concentrated hydrochloric acid 1 part, 75 per cent alcohol 99 parts); the excess of the acid is removed with distilled water, and a double staining is pro-

* "Contribution à l'étude de l'érytème tricophytique." "Arch. de physiol.," 3. sér., 1883, Bd. I, S. 171.

† "Mikrokokken bei Area Cilei." "Fortschritte der Medizin," 1883, No. 23.

duced by the use of a concentrated aqueous solution of gentian-violet, and then alcohol is used for differentiation as before.

According to Bizzozero,* the epidermis is touched with a cover-glass, which is afterward passed through a flame three times. The fat is removed by chloroform, and the preparation is stained by fuchsin or gentian-violet. For the removal of fat from the epidermic scales, they are placed for a few seconds in alcohol, then for a day or two in ether, and afterward again in alcohol. After the fat has been removed there are three methods of procedure: 1. A drop, consisting of equal parts of water and acetic acid, or of a 10 per cent solution of caustic potash, is placed upon a slide. The epidermic scales from which the fat has been removed are placed in this drop and allowed to swell, then a cover-glass is laid upon them. For the preservation of these preparations, glycerine is introduced from the edge.

2. The epidermic scale is spread out with a needle in glycerine, slightly tinged with methyl-blue. In this the fungi are stained blue, while the epidermic cells remain unstained.

3. A drop of 50 per cent acetic acid is put upon a cover-glass, and the epidermic scales, with the fat removed, are placed in it. After fifteen minutes or more, if the scales are well swollen, they are spread out with a needle; then the acetic acid is evaporated by gentle heat. (For this purpose, the cover-glass may be held high over a flame and moved to and fro.) After drying, the preparation is passed through the flame three times, and then stained for ten to thirty minutes by adding a drop of an aque-

* "Ueber die Mikrophyten der normalen Oberhaut des Menschen." Virchow's "Archiv," 1884, Bd. XCVIII, S. 441.

ous solution of methyl-violet, gentian-violet, vesuvin, methyl-blue, or of a dilute alcoholic solution of fuchsin. It is then carefully washed, dried, and preserved in balsam. Methyl-blue is preferred by Bizzozero, because only the fungous elements are stained with it. I have used most advantageously the strong alkaline solution of methyl-blue, allowing it to act for about five minutes. With this slight modification, it seems to me that this third method of Bizzozero is at present most serviceable for the determination of bacteria.

Presently I will show that in true mycosis the mycelial filaments of the mold fungus are best brought out by the method of Löffler (page 82). The presence of radiating fungi in tissue-sections can generally be determined without special preparation. But in the frequent calcification of the glands in actinomycosis it is often necessary to first decalcify, by hardening them in alcohol containing hydrochloric acid, and then to place them in absolute alcohol. The staining of Weigert* is also useful. According to this the sections are placed for one hour in a solution of orchilla (*rock moss*). Pure orchilla, which has previously lain for a long time in the air to allow the escape of the ammonia, is dissolved, according to Wedl, in such quantities, in a mixture of 20 c. cm. absolute alcohol, 5 c. cm. acetic acid, and 40 c. cm. of distilled water, that the fluid becomes dark red, and after filtering appears ruby-red. Then the section is washed in alcohol, placed in a 1 per cent solution of gentian-violet and treated as for the staining of bacteria. In these sections the nuclei are stained blue-violet, the connective tissue a pale orange, the interior of the refracting fungi a pale blue, and the outer

* Virchow's "Archiv," 1881, Bd. LXXXIV, S. 245.

parts a ruby-red, often separated by a colorless zone from the central portion.

For free amœba and cells without membranes (page 68) Brass * recommends a solution of 1 part chromic acid, 1 part platinum chloride, 1 part conc. acetic acid, and 400 to 1,000 parts of water. For hardening the tissues, in which the cells ought to be as little altered as possible (*vide* page 79), Brass recommends a $\frac{1}{8}$ to $\frac{1}{2}$ per cent solution of chromic acid, to which a few drops of a concentrated solution of the same salt is added later. After some time the preparation is placed in 30 per cent alcohol, and generally, for the complete removal of the water, in alcohol gradually made stronger, and finally in absolute alcohol. The mixture of chromic acid, platinum chloride, and acetic acid also produces excellent hardening, especially if four to six drops of 1 per cent osmic acid is added to 100 grammes of the solution. The staining is effected by borax, or ammonio-carmine, or a solution of hæmatoxylin.

* "Die Methoden bei der Untersuchung thierischer Zellen." "Zeitschrift f. wissenschaftl. Mikroskopie," 1884, S. 39.

III.

CULTURE-METHODS; PURE CULTURES.

Ehrenberg first, and later Cohn and Schröder, expressed the belief that there were true species among bacteria. On the grounds of the investigations of Cagniard-Latour and Schwann, Turpin reached the conclusion concerning fermentation, and Henle concerning the infectious diseases, that specific decompositions and diseases are produced by specific micro-organisms; and Pasteur, by inoculation experiments, proved experimentally that specific and different micro-organisms underlie specific decompositions. These views, as well as those of their opponents, who argue for inconstancy of form and action among micro-organisms, can only be conclusively proved when they are observed pure and free from all admixtures. For this reason it has been the endeavor of many investigators, for a long time, to establish methods of obtaining pure culture of bacteria, which would be free from all objections.

1. Transparent Fluid Culture-Media.

This method is the oldest, and underlies all the experiments of the earlier time on fermentation. It was used for the cultivation of bacteria by Pasteur*

* "Memoire sur la fermentation appellée lactique." "Compt. rend.," 1857, Bd. XLV, S. 913.

in his first work on the vital fermentation theory. The principle of this method, consists in transferring a particle of the original fluid containing the micro-organisms of fermentation, or a particle of yeast (which contains them in itself), to a fluid compounded to resemble as nearly as possible the original one; from this again a particle is transferred to a third solution, etc. The school of Pasteur has adhered almost exclusively to the use of the transparent fluid media, even up to the present time, often in its original form; also often with its modification, the method of dilution, later to be described.

The important morphological investigations of Cohn were also joined closely to this method.

In fluids which undergo decomposition through the action of bacteria, sometimes a diffuse cloudiness appears; sometimes precipitation occurs; more often a mouldy scum is formed. In transfer experiments a particle is transferred to a second solution from each of the portions appearing differently. This transferrence to a new solution, briefly spoken of as inoculation, is performed as follows: a platinum-needle, previously heated and cooled, straight or bent into a loop, is brought in contact with the mycoderma (the mouldy scum), or is dipped into the cloudy fluid, and then the particle, thus taken up, is introduced into the new solution. In place of the platinum-needle, a drawn-out capillary-tube can be used, with which a drop of the fluid is transferred. Such a particle or drop consists chiefly of a great mass of bacteria.

Following this inoculation, that species develops first which finds in the new solution the best conditions for its existence. When the culture-material is exhausted for one species, one or another of the forms, that at first were suppressed, may develop,

provided the particle or drop transferred contained several species. It is observed, in the course of these experiments, that small differences in conditions, such as placing the inoculated solution at a lower or higher temperature, favor the development of certain germs.

Finally, following out the conditions favorable to the development of a single germ, a more or less pure culture of this organism is obtained in some one of the transfers. But this is not always, in fact it is seldom, the organism which it is desired to isolate. Ordinarily, the result is a pure culture of one of the common *septic* species which, under the chosen conditions, supplanted the other more susceptible forms. On account of this it happened that, in the use of this method of culture, the bacterium termo and bacillus subtilis often appeared. These play a much greater part in the older bacteria literature than now.

In this way the dependence of the growth of bacteria on the culture-media was learned. This led to the choice of such fluids as would offer approximately equally favorable conditions for development to the largest possible number of micro-organisms.

Pasteur's* fluid is the oldest of these artificial media for the cultivation of bacteria. This consists of one part tartrate of ammonia, ten parts sugar, and the ashes of one part of yeast, to one hundred parts of water.

A. Mayer† used in place of the yeast-ash a solution of the salt contained in it. Then Cohn,‡ after he had used the culture-fluid of Mayer with mineral

* "Annales de Chimie et de Physique," Bd. LVIII, S. 323. Deutsch von Griessmayer, "Die Alkohol-Gährung," 1878.

† "Unters. über die Alkohol-Gährung," 1869. "Lehrbuch der Gährungs-Chemie," 3. Aufl., 1879.

‡ "Beiträge zur Biologie der Pflanzen," I, 2. Heft, S. 195.

nutrient salt, omitting the sugar, devised the following normal culture-fluid:

Calcii phosphati	·5 gramme.
Magnesii sulph. (crys.)...	·5 "
Calcii phosp. (tribas).....	·05 "
Aq. destil	100 c. cm.

In this 1 gramme ammon. tartrate was dissolved.

Naegeli* discovered that for the lower fungi and the fission fungi, nitrogen can be best assimilated if it is present as NH_2, not as well as NH, still not as well as NO, and not at all if it is in combination with other elements, such as hydrogen and oxygen, so that a descending scale may be constructed from the soluble albuminates, to ammonia and nitric acid. For carbon he arranged the following scale:

1. The forms of sugar.
2. Mannite; glycerine; the carbon group in leucin.
3. Tartaric acid; citric acid; succinic acid; the carbon group in asparagin.
4. Acetic acid; ethyl-alcohol; quinic acid.
5. Benzoic acid; salicylic acid; the carbon group in propylamin.
6. The carbon group in methylamin; phenol.

Naegeli established the following descending scale for the capacity of assimilation of nitrogen and carbon from their compounds:

1. Albumen (peptone) and sugar.
2. Leucin and sugar.
3. Tartrate of ammonia, or sal. ammon., and sugar.
4. Albumen (peptone).
5. Leucin.

* "Ernährung der niederen Pilze durch Kohlenstoff- und Stickstoffverbindungen." "Untersuchungen über niedere Pilze," 1882, S. 1.

6. Tartrate of ammonia; succinic ammonia; asparagin.

7. Acetate of ammonia.

In regard to 1, it is to be noted that the bacteria must be able to transform the albumen into peptone, and to hydrate the milk and cane-sugar, so that peptone and grape-sugar are used to the best advantage.

Naegeli recommends as the best for the mineral elements—

Potassii phos.....	1	gramme.
Magnesii sulph.....	·02	"
Calcii chlor.....	·01	"
Fluid.....	100	"

If the reaction is to be acid, Naegeli uses acid potassi phos.; for neutral and alkaline solution, potassii biphos. The poorer the nutrient elements of the N and C group are, the less concentrated the salt solution should be, while, with good nutrient elements of the N and C group, this normal degree of concentration may be exceeded. From this Naegeli devised the following normal fluids for fission fungi:

1. Aqua.....	100	c. cm.
Ammon. tart.....	1	gramme.
Potassii biphos.....	·1	"
Magnes. sulph.....	·02	"
Calcii chlor.....	01	"
2. Aqua.....	100	c. cm.
Albumen-peptone.....	1	gramme.
Potassii biphos.....	·2	"
Magnes. sulph.....	·04	"
Calcii chlor.....	·02	"
3. Aqua.....	100	c. cm.
Cane-sugar.....	3	grammes.
Ammon. tart.....	1	gramme.

Potassii biphos.....	·2	gramme.
Magnes. sulph.....	·04	"
Calcii chlor.....	·02	"

In place of the tartrate of ammonia, in the 3d formula, an equal amount of another of the ammonia salts may be used, or ·5 gramme of ammon. nitrat., or ·7 gramme of asparagin, or ·4 gramme of urea.

In place of the nutrient salts, one tenth per cent beef-extract may often be more conveniently used. For the fermentation-experiments, according to Fitz,* solutions may be employed which contain three per cent sugar, glycerine, or mannite, etc., and one tenth per cent beef-extract, to which a small quantity of calcii carb. has been added.

According to previous experiments with cultures in fluids, as was also the case in the experiments of Fitz, it is well to choose the culture-fluid (normal fluid) with special reference to the individual case. In the lack of other data at the beginning, that fluid should be selected (as was the case in the first experiments of Pasteur) in which the bacteria are observed to grow spontaneously. For micro-organisms which are observed on solid substances, a decoction or infusion of this substance is prepared: fresh soil, sweet dried fruit, hay, roots, etc. "A nutrient fluid which holds in solution such substances as in a solid state furnish naturally a pabulum upon which a fungus develops," according to Brefeld,† "in all probability will also form a suitable fluid for the development of this fungus."

Solutions for fungi are kept, as a rule, slightly

* "Ueber Spaltpilzgährung," VII. "Berichte der deutschen chemischen Gesellschaft," 1882, XV, S. 867.

† "Kulturmethoden zur Untersuchung der Pilze." "Botanische Untersuchungen über Schimmelpilze," Heft IV, 1881, S. 5

acid; for bacteria they are rendered neutral or slightly alkaline by the addition of ammonia or carbonate of soda, and then are boiled and filtered.

"In the use of a clear nutrient fluid thoroughly sterilized, in which the examination of the fungus by direct observation is made with the same ease as if they lived in clear water, the mycological investigation is turned into an algological—i. e., those conditions are artificially prepared in the nutrient solutions for the development of the fungus, under which we naturally find the algæ, as they for the most part live in water."

These words from Brefeld (*l. c.*, page 7) also forcibly point out the chief advantages of the nutrient solutions for the culture of bacteria.

These clear, usually neutral reacting, fluids must be carefully sterilized after filtration. For this purpose the sterilized cotton stopper of the sterilized flask or test-tube is removed with clean, previously heated pincettes, and laid upon its side so that it can not be soiled. Then, by the aid of a sterilized funnel or pipette, the fluid is transferred into the flask until it is about half filled, or into the test-tube until it is about one third filled. Then the stopper is again replaced. It is often desirable, in addition, to cover the mouth of the vessel with a double layer of thick filter-paper, which is held in place by a rubber band and which prevents the dust from falling directly upon the cotton. The cotton plug is proof against the admission of bacteria, but not always proof against fungi, as is to be observed when the sterilized vessels are preserved for a long time in a moist chamber.

The sterilization should be accomplished according to one of the methods described in Chapter I, preferably in most cases by the use of the steam ster-

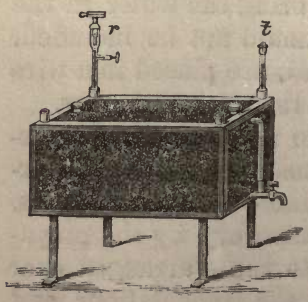
ilizing cylinder. Small volumes of fluid, as those in test-tubes, are sterilized by an exposure for a half to three quarters of an hour, larger flasks in one or two hours. The time required to bring the water in the cylinder to the boiling-point should not be included. The test-tubes, after being filled, are placed in a wire basket (Fig. 3, *d*), and then in the steam cylinder.

The manipulations with the fluids should be carried on in a place as free as possible from micro-organisms, in order to prevent infection by air-germs. This may be obtained in a laboratory by the preparation of a glass case (similar to those arranged over delicate balances), in which the inoculations and transfers are made. The walls of this case are rubbed with moist cloths, and the air is kept moist by vessels of warm water.

The inoculations are made by a platinum-needle, straight or looped, which is previously heated in a flame and again cooled, or by a capillary pipette. By means of one of these a particle or drop of the material or fluid to be used for inoculation is introduced as quickly as possible into the sterilized fluid, after removal of the cotton plug. This is then immediately replaced. If the solutions have stood for a long time without the mouths of the vessels being protected by filter-paper, so that germs of bacteria and fungi may have collected upon the cotton, it is better to burn the upper layer of cotton in a flame before removal of the plug. A large number of single experiments should be made simultaneously. The further inoculations are made in the same manner. The inoculated tubes are, according to the case, kept at the temperature of the room or exposed to a higher temperature. For the higher temperatures a brood- or culture-oven is used. This is made of metal with

double walls for the reception of water (Fig. 8), and is covered with felt or asbestos to prevent the loss of heat; or the oven is made with a third wall, and the outer chamber is filled with a layer of sand. The dimensions vary according to need; the form, quadrangular or cylindrical, is of little matter. In the quadrangular the height and breadth of the interior is about 25 cm., and the length varies from 50 to 75 cm. A thermometer (*t*) and thermo-regulator (*r*) serve for the regulation of the temperature. These last can be dispensed with if a small gas-pressure regulator is interposed between the principal supply-pipe and the culture-oven. It is heated by petroleum or gas flames, which vary in number, according to the size of the oven. These are surrounded by glass chimneys to protect them from draughts of air.

FIG. 8.



2. FRACTIONAL CULTURES.

The inoculations of fluids with pathogenic bacteria were first made by Klebs,* and the particle or drop containing bacteria ("Bakterientropfen" of other authors) was designated by him as a "fraction." Klebs proceeded in this manner (*l. c.*, page 46): "He introduced a recently drawn-out and closed pointed capillary-tube to the bottom of the fluid containing bacteria, and then broke off the point. The tube, after withdrawal, was again sealed, washed with strong alcohol, introduced into a sterilized culture-fluid, and

* "Beiträge zur Kenntniss der Mikrokokken." "Archiv für experimentelle Pathologie," Bd. I, 1873, S. 31.

was then again broken. The fluid-medium was contained in a stoppered flask and was covered with a layer of oil." This procedure was often repeated, and "in this manner it is possible to eliminate any impurities, which may be contained in the original fluid, and to obtain those organisms pure which are present in this in preponderating number."

This method has furnished no important discoveries, notwithstanding the skillful working and the sharp emphasis laid upon the development of many germs from the one originally present in preponderating numbers. In this complication of the method of Pasteur it is evident that the objection also holds good that, as a rule, after a series of fractional cultivations, the desired pathogenic organism is not present pure. But instead of this almost any form may be present which at the beginning may have been perhaps entirely overlooked on account of their small number; or else some ordinary species of septic bacteria, introduced by infection from the air or by manipulation, has been obtained pure, because, under the chosen conditions, it thrived better than the more susceptible pathogenic micro-organisms, and quickly supplanted these.

By the methods thus far described pure cultures may be finally obtained, without successfully acquiring those forms pure which it was desired to obtain. These methods are on this account only now to be used when it is desired to secure an organism in pure or quantity-culture, the source and action of which are of no importance.

3. Opaque Solid Culture-Media.

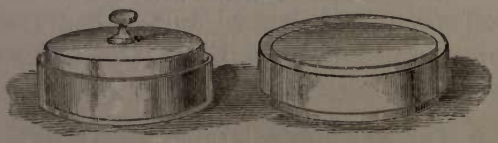
Aside from fluids, bacteria are observed developing spontaneously on solid substances. If, for exam-

ple, a slice of cooked potato is allowed to stand in the air, different slimy masses are seen to spread themselves out upon the surface of the potato. Small slimy points of different colors form upon the surface, which for some time are quite distinct from each other, but become confluent in their further growth.

These observations were first methodically made use of by Schroeder * for the pure cultures of pigment bacteria. A particle from one of these slimy points, while it is yet quite isolated, is transferred with a platinum-needle to the middle of the cut surface of a freshly cooked potato, placed in a moist chamber in order to prevent infection from the air.

The method of cultivation on potato was materially improved by Koch. The potatoes were first carefully cleaned from the coarse dirt by scrubbing, then laid for one half to one hour in a one to five per cent solution of corrosive sublimate, and finally washed in water. The potatoes, thus cleansed, with the germs adhering mostly destroyed by the sublimate, are then heated to secure certain sterilization. This is done in the steam cylinder. After reaching the boiling-point, the potatoes remain at this temperature for about an hour. While the potatoes are cooling, a moist chamber is prepared. Large bell-jars, of the form seen in Fig. 9, are cleansed by rinsing in a one

Fig. 9.



* "Ueber einige durch Bakterien gebildete Pigmente." Cohn's "Beiträge zur Biologie der Pflanzen," Bd. I, Heft II, 1872 (2. Abdruck, 1881), S. 109.

per mille solution of corrosive sublimate. Upon the bottom of the jar a number of layers of filter-paper, moistened with sterilized water or a solution of sublimate, are placed. Then the potatoes are held in the left hand, between the thumb and index finger, cut with a sterilized knife, and laid in a jar with the cut surface upward. Before this operation the hands should be washed in a one per mille solution of sublimate. The ordinary kitchen-knife is used for cutting, and, previous to use, is sterilized by heating in the flame, and while cooling is protected from the dust. For each potato a fresh knife should be used.

Then, by means of a platinum-needle which has been sterilized in the flame, a particle from one of the slimy points which is still entirely isolated, a so-called colony, is taken, and is either placed near the middle of the section of potato, or several lines of inoculation are lightly drawn entirely across the surface. In the first case the colonies develop in the middle of the section, and gradually extend toward the edge ; in the other, more or less isolated colonies develop along the lines of inoculation, which later unite and extend throughout the lines. For new inoculation only those colonies are used which are recognized as pure with the naked eye or lens, and from which a particle has been used to make a cover-glass preparation for microscopical control.

Some of the cultures can be kept at the temperature of the room, and others at a higher temperature in a culture-oven.

The great advantage of this method, as compared with the previously described methods with transparent fluid media, consists in this : namely, that each germ, whether it has been intentionally inoculated upon the surface of the potato, or is the result

of air-infection, is isolated at its point of contact with the surface, and there develops into a colony, while in fluids the different germs are mingled together.

Pure cultures may also be obtained in the fluid media, but that organism is not always present of which pure cultures are desired. In the potato-cultures it is possible to transfer the desired micro-organism, and in this way separate it from others, and after several transfers obtain pure cultures. However, the transfers must be made early, while the small colonies developed from a single germ are yet entirely isolated, and are solitary and pure, as the result of the isolation. The limitations of the method depend on the fact that potatoes do not furnish favorable conditions for the existence of all organisms, and, on this account, many species that develop on potato do not grow sufficiently to be visible. This is important, because in the solid opaque media the naked eye or the magnifying-glass can alone be used.

The inoculations on potatoes are more useful when it is desired to determine whether pure cultures obtained elsewhere, especially of pathogenic micro-organisms, possess the capacity of developing on vegetable material. Then the sterilized potatoes are inoculated in the same way with these pure cultures. The sections of the potatoes should be kept in the same manner in moist jars, and subjected to different degrees of temperature; or, as a greater precaution, a potato-section is placed upon a small glass plate, which is then lowered (by the help of a strip of nickel bent at a right angle) to the bottom of a cylindrical glass vessel about 18 cm. high and 6 cm. in diameter. The glass cylinder—previously closed by a cotton plug—the glass plate, and nickel strip should be sterilized in a dry-oven before being used.

In place of the sections of potatoes—which, on account of their yellow or white color, are used in preference to other tubers, as carrots, etc.—ground potato may be used. For this purpose, the boiled and ground potatoes are placed in flasks (preferably in the so-called Erlenmeyer flasks), and sufficient water is added to form a thick broth. This potato-broth is sterilized in the steam apparatus, and is then inoculated in the usual way with a platinum-needle. The potato-broth can be made into a very good culture-media for many bacteria by the addition of starch, sugar, peptone, and beef-extract, and can then be very advantageously used to obtain fractional-cultures of certain forms of bacteria.

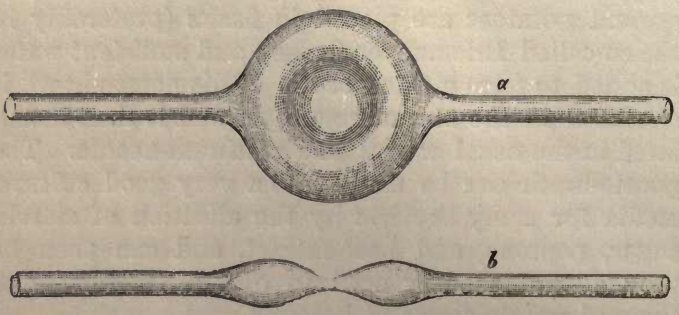
4. The Gelatin-Culture of Klebs and Brefeld.

Origin from One Germ.—Moist Chambers.—When Klebs (*l. c.*) sought to fix a coccus under the microscope in order to directly observe its division, and so follow back the entire pure culture to a single germ, he was unable to do this in a fluid-drop, first, because of the movement which occurred in the drop, and second, because after the admission of air the fluid was altered by the concentration produced by evaporation. This also changed the value of the fluid as a culture-medium. In order to prevent the evaporation of the fluid, or at least to limit it and to avoid the motion, instead of the ordinary culture-solutions, Klebs used isinglass as a culture-medium, which became stiff on cooling.

Now, in order to fix a single coccus present in the isinglass, Klebs used the chamber of von Recklinghausen and Geissler. In this (Fig. 10) a tube leads to and a second away from a middle room made of

glass of the thickness of cover-glass, the upper and lower sides of which almost touch in the middle, so

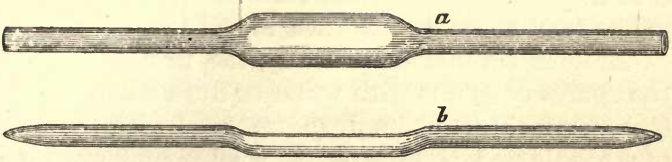
Fig. 10.



that here is formed a small capillary-chamber. If the chamber is then filled with water, culture-fluid, or liquefied gelatin, and these solutions are again poured out, a capillary-drop remains suspended in the narrow place. If these solutions at the same time contain germs, and in such numbers that each drop holds about one germ, then, in many cases, it will happen that the drop remaining will contain a germ, which can be tolerably well fixed with a high-power dry system and thus observed. Sometimes Klebs filled the entire chamber with gelatin or isinglass, so that the entrance of air to the central portion was prevented; sometimes he left only one drop in the chamber, but then took care that the air was filtered through cotton before its admission.

In other cases Klebs used, in place of the chamber with the capillary-room, a chamber the walls of which ran parallel and whose side-tubes were placed somewhat lower than the upper wall. These chambers (*b* in Fig. 11) (the description of which is after those used by myself) were filled (*l. c.*, page 46) by

FIG. 11.



allowing the excess of the fluid gelatin to run away, so that a thin layer, looking downward, covered the upper side and served for microscopical examination. Any germs fixed in this layer could be directly observed with a high dry lens or a low immersion system, and their development noted. In place of air, different gases may also be admitted.

Brefeld* sought at first to procure for fungi an entirely pure culture-medium, in order that, proceeding from a single germ, he might follow out without interruption the entire development of the fungus. These studies Pasteur† soon undertook upon yeast, and later, in 1881, Brefeld completed them for bacteria, and proved their usefulness in the phases of development of the bacillus subtilis. "By a mixture of the germs with water in such a proportion that in a certain amount, either none or only one germ is present, the separation of individual germs, even of the smallest forms, may be empirically brought about without direct observation." "There results from this method of separation an equal distribution of the individual germs in the fluid; but this is not always easy to obtain, and, if the work is not very

* (*a*) "Botanische Untersuchungen über Schimmelpilze," Bd. I, 1872, S. 10. (*b*) "Methoden zur Untersuchung der Pilze." "Verhandl. der physik. med. Gesellschaft in Würzburg," N. F., Bd. VIII, 1874–'75, S. 43. (*c*) "Kulturmethoden zur Untersuchung der Pilze." "Botanische Untersuchungen über Schimmelpilze," Bd. IV, 1881, S. 1.

† "Étude sur la bière." "Compt. rend.," 1873, S. 77.

carefully and critically undertaken, numerous foreign germs may be easily included in the culture."

Brefeld diluted empirically the fluid containing the spores or germs with water or fluid culture-media (*l. c.*, page 49) "until a drop, removed with the point of a needle and transferred to a slide and examined with a microscope, showed only one or two germs. The slide with the drop containing the germ serves as the origin for the culture, and, on this account, has received the name of 'slide-culture' to distinguish it from other forms of culture."

If now a drop of culture-fluid is added to this one spore, the uninterrupted tracing of the development is made difficult by two factors. On the one hand, the drop of culture-fluid evaporates, and, by this change in its concentration, its value as a culture-medium is diminished; and, on the other, foreign germs may gain admission. It is essential, on this account, to prevent the evaporation of the culture-drop, and to inclose it so as to prolong the possibility of the observation. "This may be accomplished in two ways: first, by alteration of the culture-fluid, and, second, by the use of a peculiar slide."

"In order to prevent evaporation (*l. c.*, page 15), caraghen or gelatin may be added to the culture-fluid in such amount, that it is still fluid at 30° or 35° C., but becomes solid when cooled to 15° C. In these gelatin solutions the fungi grow as in a thin fluid. Their development is favored rather than retarded. If now the culture can be reversed without danger, in order to prevent foreign germs from falling in it, it can be examined with a strong magnifying power." This can be done by placing it upon a cover-glass. These reversed cultures are placed in a moist jar (Fig. 9, page 102) upon a support of glass or zinc.

If it is desirable to avoid the gelatin culture-solutions, then a peculiar slide must be used, with which the evaporation of the culture-fluid and the invasion of foreign germs are impossible, without the possibility of a continuous observation being in this way in the least prejudiced.

For the observation in the hanging-drop, the slides shown in Figs. 6 and 7 may be used. A cover-glass is thoroughly cleaned by a concentrated mineral acid, alcohol, and ether. Shortly before use it is passed through the flame, cooled, and protected from dust. Then a small, flat drop of the sterilized culture-solution is placed in the center with a sterilized platinum-loop. This drop is inoculated by the introduction on a platinum-needle of a particle from a pure culture. The cover-glass is reversed, laid over the hollow slide, and a rim of vaseline drawn around it to prevent evaporation.

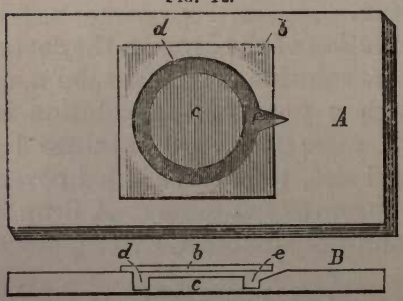
In place of the inoculation of the drop on the cover-glass, a sterilized culture-solution in a test-tube may also be inoculated with a pure culture-solution in such an amount that, after thorough mixture by shaking, a drop of equal size, tested as a dried cover-glass preparation, shows one or two germs. A drop is then taken from this and prepared on a cover-glass in the same manner.

Brefeld (*l. c.*, page 16) rejected this method. "Only a small culture-drop can be taken, since it becomes globular, and oscillates on the slightest movement; the germ-spore alters its position, and with the stronger magnifying power is scarcely accessible; in short, the observation is difficult and incomplete. This is also true if the gelatin culture-solution is used."

With the above-described precautionary measures,

after many observations with the hanging-drop arranged in this way, it may be used with great advantage (even for the smallest forms of cocci, both in the fluid and gelatin-drop), to determine whether a colony of bacteria, which with the low magnifying power appears isolated, has developed from a single germ. This has been described by Hansen,* in a remarkable manner for colonies of yeast, by the use of a suspended gelatin-drop, which was protected from evaporation. After some experience, so that this can be used conveniently, the possibility is in this way better afforded for the study of the individual phases of the development of bacteria from spore to spore (in a drop accessible to the lower systems of homogeneous immersion), and for making parallel experiments with staining for spores, which permits the fixation of these individual phases. Bienstock† even used the staining of spores directly in place of direct observation, which seems to me is going quite too far.

Fig. 12.



Prazmowski‡ used a slide (Fig. 12) to prevent the admission of air. This slide, 2½ mm. thick, possesses a circular depression (*d e*); the circular surface (*c*) inclosed by this ring is carefully ground smooth,

* "Ueber das Zählen mikroskopischer Gegenstände in der Botanik." "Zeitschrift f. wissenschaftl. Mikroskopie," 1884, Bd. I, S. 191.

† "Zeitschrift für klin. Med.," 1884, S. 1.

‡ "Untersuchungen über die Entwicklungsgeschichte und Fermentwirkung einiger Bakterien-Arten," 1880, S. 10.

and its plane lies somewhat lower (for bacteria preferably only ·5 mm.) than the surface of the slide, so that after laying on the cover-glass (*b*) between *c* and *b* there is a place for a thin layer of fluid. The circular depression extends away at *e* into a small furrow. A small drop of the sterilized solution is placed upon the surface (*c*) and is inoculated, or a drop containing an approximately definite number of bacteria is thus used. Then a cover-glass (*b*), which has previously been passed through the flame, is applied, is surrounded by vaseline or wax, so that (to prevent much evaporation) air can only enter by the furrow *e*, and then finds access to the layer of fluid inclosed between *c* and *b* from the entire circle. This layer of fluid is accessible in its entire depth to the strong dry lens or the weak immersion system. Brefeld chose, as did Klebs, the chamber (Fig. 10) of von Recklinghausen and Geissler to fix the germ, but (*l. c.*, c, 1881, page 17) the capillary-drop is, in his opinion, too deep for the smaller forms, and the amount of fluid so great, that movements are produced by the slightest influences, such as can not be prevented during the observation, and which occasion a shifting of the germs out of reach. The germ must be fixed by reducing the amount of the surrounding solution so that the movements are at a minimum; but yet the amount of the surrounding culture-solution should be sufficient for the germ to complete its full development. This is attained only in a thin layer of fluid. In order to prepare this upon the inner wall of a chamber, so that the highest lenses may penetrate it, smaller chambers must be used, which have a small capillary-room, made of glass, the thickness of the thinnest cover-glass, ground flat upon both sides so that a layer of equal thickness

is formed, in which it is possible with the strong dry lens to fix a germ for an indefinite length of time without disturbance. It is wise not to make the chamber-room larger than is necessary to meet the technical requirements.

The carefully cleaned chambers are filled full, then the contents allowed to flow out, and the single germs remaining in the thin layer of culture-fluid that adheres on the inner wall are selected. The number of the germs, which are mingled with the culture-fluid, can be far greater in this case. It is not necessary to make a previous test to determine their number, although, if they are few, prolonged search is necessary to find them on the wall. I was successful without much trouble in observing for an unlimited time the germs of bacilli and other bacteria, and in following thus a complete series of development as with the greater fungi. In this way it is easily possible to determine the length of time which is necessary for the process of growth, for division, and finally for the entire cycle of development from spore to spore.

The value of these chambers for the examination of the fission fungi extends even to the smallest forms, which are still easily accessible to the observer with the strongest dry system.

Brefeld used chambers of the form *a* (Fig. 11), designed by Geissler. I used the form *b*, also designed by Geissler. As was shown by my experiments, it is possible to prepare a thin layer of fluid in these chambers, if they have been carefully cleaned with mineral acid, alcohol, and ether, and have been sterilized. In place of the fluid, it is also advantageous to prepare a thin layer of gelatin, in similar chambers as was done by Klebs in 1873. In such

layers of gelatin I have traced, for single species, the formation of colonies, visible to the naked eye, which had developed from one germ. In the chamber chosen by myself, a one twelfth homogeneous immersion-lens with a low ocular could be used.

With all these chambers the ordinary warm stage is, as a rule, necessary. Up to this time, the chambers with parallel walls have been used by most observers for the uninterrupted observation of the cycle from spore to spore. For experimental work, I prefer the far more convenient mode of observation by means of the hanging-drop.

5. Method of Dilution.

Although Brefeld had positively shown concerning mould fungi, and Pasteur concerning yeast, that, by the systematic dilution of a solution containing micro-organisms or spores, a determined quantity of fluid may be prepared so as to contain approximately one germ, with which experiments can be made by transfers to sterilized media, Naegeli,* in 1877, notwithstanding all the previous experiments, denied the possibility of obtaining pure cultures of bacteria. "Fission fungi absolutely permit of no pure culture, in part on account of their extraordinary minuteness, in part on account of their general diffusion in the water and air." Naegeli then gave a method of dilution, not that he wished to make hopeless experiments to obtain pure cultures (as was stated in his communication at that time), but in order to prove the capacity for multiplication of the fission fungi, depending upon the degree of alteration of the medium. In this method he diluted a fluid containing

* Naegeli und Schwendener, "Das Mikroskop," 2. Aufl., 1877, S. 644.

a certain number of bacteria with a definite quantity of sterilized water. Later, Naegeli himself came to the conclusion (*l. c.*, page 646) that the solution of this problem of equalization could only be arrived at by testing. He states that he then conducted such empirical experiments in his studies of the lower fungi,* beginning in 1871. In these experiments, he diluted decomposing urine so greatly with water that each two drops contained one germ. By transfers of a drop to a glass containing a sterilized solution, he then succeeded in obtaining a certain separation of the rods and cocci. After 1877 he denied the possibility of obtaining pure cultures.

I find in Naegeli's investigations, unfortunately, no statements to bring into harmony these later communicated results of 1871, with his complete denial of 1877, and his theoretical views concerning the morphology of bacteria and the origin of all bacteria from cocci. Certainly without any knowledge of these experiments of Naegeli in 1871, Lister† in 1878 stated that he had so diluted sour milk that a drop contained one germ. He now inoculated sterilized milk with from two to four drops of the diluted fluid, and constantly obtained a souring and coagulation; less certainly, however, if he used only one drop, because in this case not every drop in reality contained a germ.

Fitz‡ also used in his experiments on fermentation the method of dilution which he alone designated as the "one-cell culture."

* 1882, S. 13.

† "On the Lactic Fermentation and its Bearings on Pathology." "Transactions of the Pathological Society of London," 1878, vol. XXIX.

‡ "Ueber Spaltpilzgährungen," VII. "Berichte der deutschen chemischen Gesellschaft," Bd. XV, 1882, S. 867.

"In order to obtain a pure culture of a fission fungus which causes fermentation, it is absolutely necessary to start out from a single cell as seed. In an ordinary impure culture, with the aid of a counting-chamber, the number of fission fungi contained in one drop is approximately determined; then this drop is so greatly diluted with sterilized distilled water that, in from five to ten drops of the diluted, well-mixed fluid, only one fission-fungus cell is present; then a drop is added to each of a series of about fifty flasks, filled with fluid and sterilized, and these are then placed in a thermostat at 37° C. Of the fifty, five or ten, in the course of the next three weeks, show the development of fungi. In each one of these flasks the culture-fungus is solitary and pure, because it has developed from a single cell. Thus the different fission fungi which were contained in the unclean culture are isolated."

For pathogenic bacteria, especially for the anthrax bacilli, Buchner* used the method of dilution in which the spleen-pulp is scraped and diluted so much with sterilized water, that a single bacterium is present in about 10 c. cm. Then the sterilized culture-solutions are infected with 10 c. cm. of the fluid. Hansen† proceeded in a similar manner. He made the dilution for yeast so that 2 c. cm. contained one cell.

For determining also the number of germs in the original fluid as well as in the diluted solution, a definite amount is placed in an apparatus for numbering red blood-corpuscles. For this the chamber of Hay-

* "Ueber die experimentelle Erzeugung des Milzbrandcontagiums aus den Heupilzen." "Untersuchungen über niedere Pilze von Naegeli," 1882, S. 147.

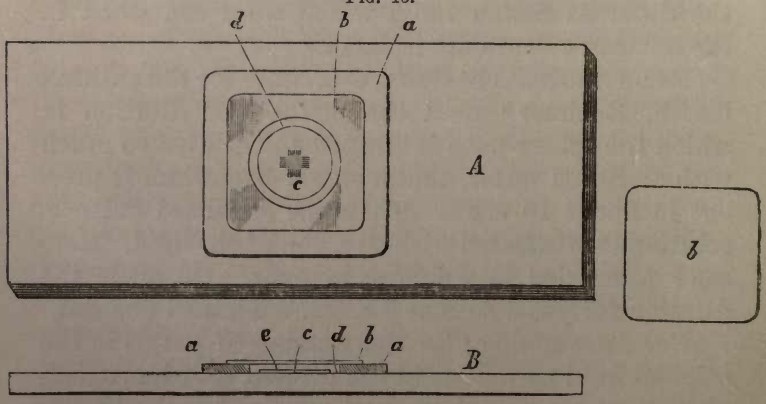
† "Ueber das Zählen mikroskopischer Gegenstände in der Botanik." "Zeitschrift f. wissenschaftl. Mikroskopie," Bd. I, 1884, S. 191.

em-Nachet may be used. This (Fig. 7, page 40) consists of a slide (*A B*) upon which rests a glass plate (*b*) provided with a circular aperture (*c*).

This glass plate is of a definite thickness, ·2 mm., or for bacteria still better, ·1 mm., as is furnished by Zeiss. By application of a very carefully ground cover-glass (*d*), a room is formed, bounded by parallel walls, the height of which is exactly ·1 or ·2 mm., and it will contain a definite amount, a unit of volume, of the fluid. The numeration is accomplished by the aid of a crossed ocular micrometer. The fluid must exactly fill the room, and the cover-glass should fit accurately.

A modification of this (Fig. 13), which was designed by Thoma,* permits of yet finer work. This is

Fig. 13.



manufactured by Zeiss. Upon a slide (*A B*) an even polished glass plate (*a*) is fastened, whose circular

* Abbé, "Ueber Blutkörperzählung." "Sitzungsberichte der Jenaischen Gesellsch. f. Med. u. Naturwissenschaft," 1878, No. 29. Lyon und Thoma, "Ueber die Methode der Blutkörperzählung." Virchow's "Archiv," 1881, Bd. LXXXIV, S. 131.

aperture (*d*) forms the side-walls of the chamber. In this aperture a circular glass plate (*c*) is cemented, which is so thick that the room (*e*) between it and the superimposed cover-glass (*b*) is exactly ·1 mm. high. This is the real counting-room; *a* and *c* are carefully ground parallel to the surface of the slide; in the same way the cover-glass (*b*) must be carefully ground into a parallel plane, and so cleaned that, by its joining with the polished upper surface of the chamber, it forms Newton's color-rings, which remain after the removal of the pressure. The mixture is made in a mixing-vessel, which is to be also used for the first numbering-chamber. Since exact instructions as to its use are given with the apparatus, these data suffice. The advantage of the Thoma chamber is that the ocular micrometer is omitted, because on the plate (*c*) a crossed division is engraved which has one q. mm. divided into four hundred quadratic equal fields.

In this way the absolute value of a portion is determined, which is not possible when this is altered by each objective and each length of the draw-tube, as in an ocular micrometer. But, if the numbering is made ever so exactly, and the mixture also carefully prepared, still an absolute certainty for the source of a germ is not reached, either in the method of dilution or the "one-cell culture." The error also belongs to this method, as Hansen in his excellent communication frankly states, viz., that it is not certain whether the number of cells are really present in the prepared inoculation-fluid which in the beginning are computed to be present. It may also occur that not a single cell is included, or that several are present, as was desired.

The method of dilution also, in its best form, can only be conditionally adapted to the theoretical post-

ulate of the origin from one germ. If, further, only an approximate guarantee is desired, that the different forms of bacteria contained in a mixture are moderately separated by the method of dilution, large numbers of individual experiments must be made. The number of these was placed at fifty by Fitz, for his individual case; but there must be hundreds and thousands if decomposing fluids, or a very dirty water or similar mixtures containing many different bacteria, are employed.

All investigators, who have made use of the method of dilution (on account of the limitations of the method, but without the admission of the practical points causing them), have taken care to previously prepare the cleanest possible inoculation material by the quantity-culture, with the aid of such secondary means as are dependent, according to Brefeld (*l. c.*, c, 1881, page 12), on the varying modes of life and certain morphological and physiological peculiarities of the different forms. With these restrictions, the continual dilution, even to a "one-cell culture," is very useful for individual pathogenic organisms, still better for ferment bacteria, the conditions of whose existence are already approximately known, or suspected from the manner of their spontaneous presence. The fact first to be determined through pure cultures—viz., the biology of bacteria—is in these cases provisory, since it is presupposed that it is already known.

Culture-fluids are chosen in which it is certainly known, or presumed, that the respective fermentation can proceed. These are inoculated with a particle or drop of the original impure material; then the flasks thus inoculated are placed at a higher or lower temperature; transfers are made a second time

after development of the germs has occurred, and so on until an approximately pure culture of an organism is obtained, as in methods 1 and 2.

Other flasks are kept free from air, if it is supposed that the fermentation under consideration proceeds* better, with a restriction or absence of the oxygen of the air. In the anaërobic experiments this will be more particularly detailed. After a few of these transfers, enumeration and dilution are made, and then the sterilized culture-solutions are inoculated with the unit of volume containing the one germ.

METHOD OF ISOLATION BY HEAT.

With these measure-cultures belongs a more exact description of the "heating method," as it may be briefly designated.

In all cases in which bacterial development occurs, after a shorter or longer exposure to a boiling temperature, or to a still higher temperature (as was first shown by Cohn),† it is always due to the presence of spores forming bacteria, and to the resistance of the spores for a varying length of time to the high temperature. Miquel ‡ isolated from urine a micro-organism bacillus ureæ (that forms ammonia), by heating a glass of water, that contained its spores, to a temperature of 108° C. Brefeld # found that, for

* Fitz, "Ueber Spaltpilzgährungen," IX. "Berichte der deutschen chemischen Gesellschaft," 1884, Bd. XVII, S. 1188.

† "Untersuchungen über Bakterien," Bd. IV. "Die Bakterien und die Urzeugung." "Beiträge zur Biologie der Pflangen," Bd. II, Heft 2, 1876, S. 249. Vergl. auch die Literatur im ersten Abschnitt und über Sporenfärbung.

‡ "Bulletin de la société chimique de Paris," 1879, Bd. XXXII, S. 127.

"Botanische Untersuchungen über Schimmelpilze," Bd. IV, 1881, S. 51.

the destruction of the spores of the bacillus subtilis, exposure to a boiling temperature for three hours was necessary, or to a temperature in the oil-bath of 105° C. for fifteen minutes, or 107° C. for ten minutes, or 110° C. for five minutes. By reducing this time, these bacilli can be obtained in cultures, pure from all less resistant micro-organisms. Prazmowski* boiled fluids containing spores for a longer or shorter time, to gain and retain pure cultures of bacilli and clostridii, which form spores. Without reference to these investigations, Gunning,† proceeding from a mistaken conception of the method of culture with solid transparent media, has put forward the systematic use of higher temperatures for the isolation of bacteria. Gunning himself in this way also, as all before him had done, isolated spore-forming species.

In systematically testing this method by the use of pure cultures of bacteria (both those forming spores and those free from them), I discovered what was to be confidently expected from the biology of bacteria—namely, that, by systematic heating below, to, and above the boiling point, only the more resistant forms can be separated from the less resistant. Consequently the use of this method is limited, for practical purposes, to the separation of spore-containing bacteria from those free from spores. For this, it is to be recommended. If there are two kinds of spore-forming species present having approximately the same resisting power, it is not possible to obtain, by a shorter or longer heating alone, a sufficient separation. The process of obtaining by heat a tolerably

* "Untersuchungen über die Entwicklungsgeschichte und Fermentwirkung einiger Bakterien-Arten," 1880, S. 8.

† "Beiträge zur hygienische Untersuchung des Wassers." "Archiv für Hygiene," Bd. I, 1883, S. 335.

or quite pure culture is, as I have previously stated,* restricted to certain cases; but for these it is very useful—e. g., it is the best method for obtaining from the prepared quantity-cultures a separation of the spore-containing bacteria from the spore-free forms. Often in this way a really pure culture may be obtained.

6. Cultures in Capillary-Tubes, after Salomonsen.

Salomonsen† observed in spontaneous changes in the blood, caused by putrefaction, that black spots of decomposition appeared (formed by the reduction of the oxyhæmoglobin), which, on the bottom of the vessel, possessed a sharp circular contour, and higher up a more club-shaped form. These gradually increased in size, became confluent, and then produced a diffuse dark color in the entire mass of blood. While these spots are still isolated, according to Salomonsen, each consists of a single form of bacteria, which by its growth brings about the alterations in the color of the blood. Each such spot is a colony, a genuine pure culture of a single species.

If now fresh or defibrinated blood is drawn into a capillary-tube, these isolated decomposing spots also appear in the blood in these tubes. In place of the true capillary-tube, lymph-tubes or fine glass-tubes can be used, which are drawn out at one end to a capillary-point (Fig. 14).

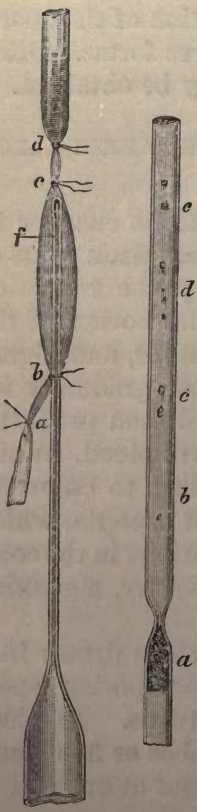
Among the colonies thus developed with a low

* "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. II, 1884, S. 330.

† "Zur Isolation differentur Bakterienformen." "Botanische Zeitung," 1876, No. 39. "Studier over Blodets Foraadnilse," 1877. "Eine einfache Methode zur Reinkultur verschiedener Fäulnissbakterien." "Botanische Zeitung," 1880, No. 28.

magnifying power (such as a simple lens), small differences are observed in the size, the rapidity of development, and in the form (*b*, *c*, *d*, *e*, of the second cut, Fig. 14). Each of these small differences is a visible indication that these different colonies have their origin from different forms of bacteria.

Fig. 14.



If it is now desired to transfer such a pure culture, then the tube is broken near the colony; a previously sterilized platinum-needle is introduced, and it is rapidly transferred to a sterilized solution.

The tubes possess at one end a slight constriction or indentation, at which point (*a*) the tube is closed with cotton or asbestos, while the end drawn out to a capillary-point is sealed. The tube thus prepared is sterilized by heat. The closed end is first broken in the fluid, then the tube is filled by sucking on the end plugged with cotton (or asbestos); after its withdrawal it is cleaned with alcohol, and then freshly sealed at the capillary end or closed with varnish. The appearance of isolated decomposing spots, or, in general, of colonies of bacteria, is similar in substances, which spontaneously solidify or gelatinize (as in blood by coagulation); while in true solutions such a sharp isolation of the different colonies does not occur even in the capillary-tubes.

In regard to this point, then not completely understood by Salomonsen, this method was one of the most reliable for producing real pure cultures, until a few years ago.

If blood is taken directly from the vessels of sound animals without letting the air come in contact with it, such decomposition-spots never occur. According to Salomonsen, it is carried out in the following manner (first part of Fig. 14): the end of a sterilized glass tube, that has been drawn out and sealed, is introduced into the vessel which has been previously laid bare, and opened under antiseptic precautions. After the blood in the tube has first flowed out, it is bound fast to the vessel at *b*. Then the sealed end is broken within the vessel at *f*, and the tube filled by suction at the opposite end, which has been plugged with sterilized cotton or asbestos. After this the ligatures *a*, *c*, and *d* are tightened, the vessel is cut between *a* and *b*, and between *c* and *d*, and the tube is sealed in the flame below *b*. At this end, at no time during the filling of the tube can air gain admission, and at the other end only filtered air enters. The tube is then placed in a thermostat at the temperature of the blood.

The glass tubes are first disinfected with a one per mille solution of sublimate, the sublimate removed by alcohol, the alcohol by ether, and the last evaporated by warming. This method I have found to be the most reliable one. This disinfection should be performed before the tubes have received their definite form; then one end is drawn out and sealed; the other end receives a slight narrowing or twisting, and here, as at *a* (second part of Fig. 14), it is securely filled with asbestos or cotton. The tubes, thus prepared, are finally sterilized by exposure to a tempera-

ture of 150° or 160° C. for one or two hours, and after cooling, immediately before the operation, the capillary end is passed once more quickly through the flame.

Zahn,* in place of such glass tubes, uses a pipette that will contain from 50 to 100 c. cm., one end of which is drawn out to a point, and the other has previously received a constriction at one point. Then the balloon of the pipette is heated in the water-bath or in a flame. During the manifold heating the air is displaced by oxygen, carbonic acid, or hydrogen, and then, during this process of heating and the consequent expansion of the gas, both the ends are sealed, the second one at the point of constriction. The sterilization is accomplished by exposure to a high temperature. Since the air in the pipette is expanded by the heating, after breaking the point in the vessel the filling of the pipette follows from the negative pressure. In the subsequent sealing, according to Zahn, fine cracks sometimes occur, on account of which great care must be taken. I cover over these cracks, immediately after sealing the ends, a layer of sealing-wax. If the blood is taken from sound animals, the clot separates from the blood-serum. Upon the latter there forms sometimes a fine scum of the smallest fat-drops and altered blood-corpuscles. The cellular elements gradually disintegrate by retrograde metamorphosis. The granulations disappear, but true bacteria or cocci do not gain an entrance, and the spots of decomposition do not appear. These blood-granulations, like the bacteria, are immediately apparent, and also, like bacteria, become cleared up, by anamorphosis of the protoplasm.

* "Untersuchungen über das Vorkommen von Fäulnisskeimen im Blut gesunder Thiere." "Arch. f. pathol. Anatomie," 1884, Bd. CXLV, S. 401.

If the blood contains bacteria, as is the case in many infectious diseases, pure cultures of these may be obtained in the tubes in this way.

7. The Infection-Methods.

In the consideration of the strongly obligatory parasites, those referred to in the Introduction, as possessing in the highest possible degree adaptability to the parasitic mode of life, only the tissues of the animal or vegetable organism would seem *a priori* to offer the necessary conditions for their existence, and in many extreme cases only a certain form, or even only a certain variety of these tissues, seems to be able to serve as host to the parasite. These observations lead to the inoculation of susceptible species of healthy animals and plants with the parasites, so that the artificially infected organism may thus contain these parasites in a pure condition. I will only mention the known experiments concerning trichinosis, and the infection of plants by de Bary, van Tieghem, and especially by Brefeld, who in this way more exactly defined the problems to be solved by the infection-method *—i. e., "first, the determination of how and where the germs of the fungus gained admission; then, secondly, the tracing of the development of the fungus, and the continuous extension of the typical disease in the host from the action of the germs that have gained admission."

The transmissibility of many infectious diseases, established both by clinical observation and the facts of epidemiology, as well as by numerous experiments made after we had learned to ascribe many of these diseases to micro-organisms, led to the use of the

* "Die künstliche Kultur parasitischer Pilze." "Botanische Untersuchungen über Hefenpilze," Bd. V, 1883, S. 1.

method of infection in the study of bacteria. We must here forcibly distinguish two things: first, the transfer to animals of pure cultures of bacteria derived elsewhere for the determination of the malignant peculiarities of these germs; and, secondly, the transfer from animal to animal without previously obtaining pure cultures elsewhere. For such infection, almost entirely formerly, and also largely now, the ordinary species of animals have been used, which were ready at hand in the laboratory. Koch* has shown that in the inoculation of field- and house-mice with a decomposing fluid, only the latter died of a certain form of septicæmia caused by a fine bacillus, and that, after several transfers from mouse to mouse, the blood of the last animal presented an absolutely pure culture of this form of bacillus. He demonstrated the same fact for another form of bacillus by the inoculation of rabbits with other decomposing fluids. In these experiments also, after a few transfers from animal to animal, all other forms of bacteria originally present in the fluid in large quantity were eliminated, and the blood contained a pure culture of a single form of bacteria. Anthrax bacilli kill mice with such absolute certainty that, by the inoculation of mice with mixtures of bacteria which contain the anthrax bacilli, after a few transfers these bacteria can be obtained pure. Further, Carter and Koch succeeded in infecting monkeys with the blood of relapsing fever, so that the blood of these animals presented pure cultures of the spirochætæ of this disease.

* "Untersuchungen über die Aetiologie der Wundinfectionskrankheiten," 1878. "Zur Untersuchung von pathogenen Organismen." "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. I, 1881, S. 1.

From these observations Koch deducted the important principle that for infection-experiments, first of all, such species of animals should be used as are proved to be susceptible to the disease under consideration; and that animals should be chosen of the same species as that in which the disease occurs. If this is impracticable, the species which resembles most closely the form of animal in which the disease spontaneously appears should be used. The technical rules to be observed are given later in the inoculation-experiments.

These infection-methods are to be used to obtain pure cultures in those cases where the blood or the artificially infected organism in general presents a pure culture of an infectious micro-organism, which shows in the highest degree parasitic adaptability, and also in the study of all those infectious diseases in which micro-organisms have not yet been found, but which clinically and epidemiologically appear to be purely contagious. But in some of these diseases, in many acute exanthemata, such inoculations seem quite purposeless, because these diseases are most probably confined exclusively to man, and because the supposed micro-organisms probably find, in the human body only, the conditions necessary for their existence. In such cases, naturally the solution of all the questions presented in the Introduction is impossible, and it would be unreasonable to ask here, from the bacteriological investigation, the solution of the problems, which are insoluble from the nature of the case.

But in these cases those portions of the problems which can be solved must be the more certainly determined, and the clinical and epidemiological observations the more critically studied. But bacteriology

must only first forego the solution of all problems, when all possibilities are really exhausted; for an improvement in the methods sometimes renders possible the complete solution of problems seemingly insoluble. As a most remarkable example of this, Koch's inquiry into the ætiology of tuberculosis may be cited, which showed that the experimental pure culture of the bacillus tuberculosis was possible outside of the animal organism, in spite of the apparently intense degree of its parasitic adaptation.

8. The Cultures upon Transparent Solid Nutrient Media according to Koch.

In the communication upon this method * the means were given, which had been previously approved of for the re-establishment of pure cultures in certain cases.

1. The advantages of the solid opaque media for the isolated culture of the characteristic pigment bacteria, after Schroeter.

2. The possibility of the isolated development of pure colonies of bacteria in blood, and of the differential diagnosis of such colonies with a low magnifying power, after Salomonsen.

3. The advantages of transparent fluid media for many cases, according to Pasteur, Cohn, and Brefeld.

4. The principle of the development from one germ, after Brefeld, Pasteur, Lister, Naegeli, and Fitz.

5. In the use of this principle the necessity of the local separation, in order to give to each individual germ the possibility of development, isolated and pure.

* "Zur Untersuchung von pathogenen Organismen." "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. I, 1881, S. 1.

6. The introduction of gelatin by Klebs and Brefeld, to prevent the evaporation of the culture-fluids.

The advantages of these different methods had only been attained separately, even by Koch, but he found the connecting link which permitted the union of most of them, and by this combination a most universal method, and at the same time the most simple of all methods, resulted.

Upon solid culture-media, germs, which have been intentionally deposited there or have been derived from the air, develop into isolated colonies. If this solid nutrient medium is opaque, a satisfactory observation is only possible in the case of micro-organisms having a peculiarly characteristic growth—as, e. g., pigment-bacteria. But if the culture-medium is not only solid, but likewise transparent, colonies can be differentiated from one another by the help of a low magnifying power, through peculiarities of their growth, which are not perceptible to the naked eye, or a simple lens. Koch united the advantages of the solid culture-media for the separation of different germs to the advantages which the transparent media possess for direct microscopical observation. This simultaneous emphasis upon the solidity and transparency of the culture-medium distinguishes Koch's method from all others.

Koch followed two quite different ways in attaining these objects—viz.: he first chose a solid, transparent culture-medium which, without any additions, would answer to these requirements; and, second, he produced a solidification of the ordinary clear nutrient solutions by the addition of gelatinizing substances. The addition of these gelatinizing substances to the culture-media led to the discovery of the principle of solidity and transparency, and the

media thus prepared temporarily preceded the natural solid transparent media. The former were made by the addition to the previously known normal culture-media, and to the approved decoctions and infusions, of sufficient gelatin to convert these solutions, at the temperature of the room, into transparent solid, nutrient substances.

Koch found, if he inoculated such a nutrient gelatin, while it was still liquid, with a drop of fluid containing bacteria, that in the subsequent solidification the individual germs were each surrounded by a layer of gelatin. If the fluid used for inoculation did not contain too many germs, these remained sufficiently separated after the gelatin became solid, so that each germ was isolated at the point of its fixation, and could there develop into an isolated colony. If, now, the gelatin solution was allowed to solidify on a transparent glass plate, the development of the colonies from single germs could be observed with the microscope before they were perceptible to the naked eye or the simple lens.

Koch did not use the gelatinizing substances, as did Klebs and Brefeld, to prevent evaporation. The better nutrient capacity was not a desirable quality, as Brefeld emphasized, but was often even undesirable, or at least an indifferent one. The possibility of the reversal of the nutrient gelatin-drop to prevent the air-infection, also advanced by Brefeld as desirable, was quite unnecessary; because, upon the solid culture-media, the germs originating from the air were also strictly localized in their development, so that by their position they could be easily distinguished from the colonies of the germs intentionally inoculated. To this is added the further difference that both Klebs and Brefeld, in order to use the gela-

tinizing substances in their sense, must previously have a pure culture, which the first obtained by the fractional culture-method, and the second by the method of dilution. Neither in the writings of Klebs nor of Brefeld is there found the slightest intimation of an experiment, or even of an idea of using gelatin to solidify the solutions for separation of different germs, or for obtaining pure cultures, as was clearly put forward by Koch.*

In a colony composed of very similar organisms, recognized as growing characteristically, first by the microscope and later by the naked eye, the peculiarities of each organism are grouped together. The whole habitat of a colony can in this way be especially valuable for the differential diagnosis of organisms, the forms of which are very similar. The study of the morphological differences, which show themselves in the pure colonies, makes this bacteriological method especially useful for hygienic purposes.

The proof that a colony, visible with the naked eye and with a low magnifying power (up to 80 or 120 diameters), which has a representative and characteristic growth, has developed from a single germ, was not certainly determined before the discovery of this method, and was later strikingly brought out by Hansen (page 110). After some practice, the observer learns to determine whether a colony originated from a single germ, or whether it has proceeded from the union of several colonies.

* When Zopf, in his work concerning the fission fungi, referred to Koch's methods under Brefeld's methods of gelatin-culture, he paid no regard to the statements of either investigator, since there can scarcely be presented greater differences than exist between these two methods.

A. TRANSPARENT SOLID MEDIA MADE BY THE ADDITION OF GELATINIZING SUBSTANCES—"NUTRIENT GELATIN."

PREPARATION OF NUTRIENT GELATIN.—To one of the tested nutrient solutions, decoction, or infusion, there is added pure, finely cut gelatin, preferably for the test-tube cultures 5 per cent in amount; but for the slide- and especially the plate-cultures, 10 per cent. This gelatin is allowed to soak for half an hour to one hour, then is completely dissolved by application of heat. Since the reaction of gelatin is acid, and most bacteria require either a neutral or slightly alkaline reaction for their growth, the warm gelatin solution is neutralized, or, still better, for most cases, rendered slightly alkaline with carbonate of sodium.

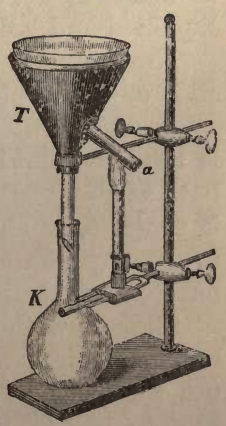
The neutralized gelatin solution is then boiled for about one hour on the water-bath, for the complete separation of the neutralization precipitate and all other substances coagulable by heat; and after this, while still hot, is filtered through a moist fluted filter. The filtrate, after renewed boiling, must, when cold, be solid and clear without any cloudiness. A transient cloudiness produced in the cooking by the phosphates, and which disappears after cooling, has no significance.

It is self-evident that no nutrient gelatin offers to all bacteria equally favorable conditions for existence; but still it is desirable to possess gelatinized solutions, which are as universally valuable as possible, so that they furnish, to the largest possible number of forms of bacteria, conditions sufficiently favorable, so that the germs can develop into perceptible and separable colonies. The meat-water peptone gela-

tin, as described by Löffler,* supplies this. It is prepared in the following manner: half a kilogramme of good finely chopped meat is added to one litre of distilled water, well stirred up and allowed to stand in an ice-chest twenty-four hours. Then this is filtered through gauze, finally pressed out with a peculiar meat-press, and the volume of the fluid, by the addition of distilled water, is restored to one litre. To this meat-water is added 10 grammes of dry peptone, 5 grammes of chloride of sodium, and 50 to 100 grammes of the purest gelatin. The swelling and solution of the gelatin, neutralization, boiling, and filtration are performed as described above.

I have obtained the same object in a more simple manner with a nutrient gelatin, which consists of peptone 3 per cent, grape- or cane-sugar ½ per cent, and beef extract ½ per cent, with 5 to 10 per cent of gelatin. The addition of a small quantity of sugar to this otherwise much less advantageous solution makes it available for the same purposes. The boiled and neutralized nutrient gelatin must be filtered while hot. A hot-water filter (Fig. 15, *T*) is most conveniently used for this purpose, in which the mantel of water between the glass funnel and the outer copper wall is kept warm by a flame which is placed under the tube (*a*), the lumen of which communicates with the water-mantel. It can be done less conveniently by filtering successively small

Fig. 15.



* "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. I, 1881, S. 169.

portions while hot, during which it is advantageous, from time to time, to carefully warm the funnel by a small flame.

The neutral, clear, nutrient gelatin is then poured into sterilized test-tubes to about one third their height (equal to about 10 c. cm.), with the aid of a sterilized funnel or pipette, and the sterilized cotton stoppers after this are again replaced. This nutrient gelatin in the test-tubes is sterilized by discontinuous boiling. For this purpose the solidified gelatin in the test-tubes may be carefully liquefied directly in the flame, and then boiled, or the gelatin may first be dissolved by placing the tubes in warm water, and then, after drying the glass, it may be boiled for a short time in the flame, or this may be done in the water-bath. This should be repeated for about ten minutes for four or five successive days. If the gelatin has stood for a long time, so that it has begun to decrease by evaporation, it must be once more liquefied and boiled before using.

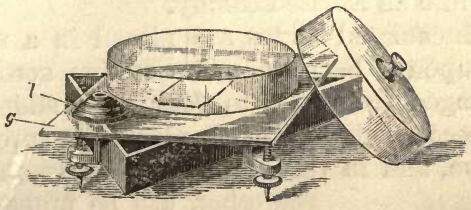
The nutrient gelatin thus prepared is used for slide-cultures, plate-cultures, and test-tube cultures.

a. Slide-Cultures.—The gelatin in the test-tubes is rendered fluid by heating or warming in a water-bath at about 30° C., and the cotton stopper, so far as it projects, is charred in the flame before opening, to destroy whatever germs of fungi or bacteria may have gathered upon it.

Slides are thoroughly cleaned with mineral acid, water, alcohol, and ether, and sterilized by exposure for one or two hours to a temperature of from 150° to 160° C. in a dry-oven. For this purpose a number of clean slides are placed in a small beaker, over which a larger one is turned to protect them from dust while cooling. For the reception of the

gelatin a number of slides are placed, as nearly horizontal as possible, on a table or glass plate, and covered with a glass jar to protect them from dust. For this purpose the apparatus (Fig. 16) is most conven-

Fig. 16.



ient. This consists of a triangle made of wood, provided with leveling-screws, and upon the triangle a ground-glass plate (*g*) is laid. This plate, with the aid of a level (*l*) and the screws, is made horizontal. Under the glass plate there is room to place a dish with ice-water, in order to thoroughly cool the glass plate, and thus the solidification of the gelatin is accelerated.

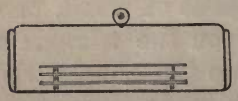
With a sterilized pipette the fluid gelatin is placed upon the slide in the form of a long, thin layer, which is a few millimetres thick, and does not extend at any point to the edge of the slide. If it is not possible to arrange the slide exactly horizontal, then the consequent difficulty can be avoided by dipping the test-tube in cool water, or holding it under a water-jet, so that the gelatin is so far cooled that it is no longer a thin fluid, but has more consistency.

When these layers of gelatin have so far solidified as to render the gelatin not completely solid, but very consistent, then they are inoculated with a sterilized platinum needle, with which a particle of the infecting substance or fluid has been taken up. With this, from three to five lines of inoculation are

lightly drawn upon the layer of gelatin. The depth of these lines should not extend to the slide. When the gelatin is completely solidified the germs become fixed, and, if there are not too many present in one line, each is so far separated from others that it may develop into an isolated colony.

The inoculated slides are placed in a moist jar (Fig. 9) upon a bench of glass or zinc, across which are arranged from two to four such slides. Over these a second glass bench is placed for support and protection, and in this way several tiers, one above the other, can be prepared (Fig. 17). These glass benches are carefully cleaned and sterilized by heat before use. Such benches can be prepared from a strip of zinc about 4 cm. broad and 14 cm. long, each end of which, from 1 to 1½ cm. wide, is bent over, or from a strip of glass 14 cm. long and 4 cm. broad, by cementing to the ends small glass supports with Canada balsam. Upon these benches, strips of dry filter-paper of corresponding size are laid, and upon them the slides are placed. An absolute protection against infection from the air is not essential. The germs in the air can only settle upon the surface of the gelatin, and are in this way recognizable, even if by chance they develop into a colony upon a line of inoculation or in the immediate vicinity. The growth of a colony in the line of inoculation is observed with a dry lens of low power, magnifying from 80 to 150 diameters. If different forms of colonies develop in the line of inoculation, a second inoculation is made with the aid of a low magnifying power of 15 to 20 diameters for the removal of a colony. This transfer is made with each colony,

Fig. 17.



showing differences in growth, to other slides, so that after a few transfers there is only a single form upon each slide.

In all gelatin cultures the following should be noted: In respect to their behavior toward the gelatin, all bacteria can be practically divided into those which leave the gelatin solid and those which liquefy it. The first develop in the interior of the gelatin in a round, oval, or disk form, etc., while on the surface a characteristic superficial growth is produced, sometimes in the form of a sharply circumscribed circle, sometimes in leaf- or grape-like arrangement, sometimes in the form of concentric rings, etc. Transfers of such bacteria should be made both from the isolated colonies in the interior and from the edge of the colonies developing superficially.

The bacteria producing liquefaction of the gelatin do this in different ways—sometimes rapidly, sometimes only slowly, in the form of a funnel. Since, with the advance of the liquefaction, the advantages of the solid culture-media are lost, these forms should be separated from each other as quickly as possible by using the fewest germs possible for inoculation, so that the liquefying colonies do not come in contact with the others until they have developed sufficiently for further inoculation. The inoculation should always be made from the edge of the colony where the liquefaction is extending upon the still solid gelatin, because in the interior of the liquefied portion a mixture with other forms may have taken place already. Colonies should be chosen for inoculation which, in the microscopical examination with a dry objective, present a uniform appearance throughout, and in which the characteristic differences are as clearly pronounced as possible. In addition to this, for con-

trol, it is necessary to make dried cover-glass preparations for the microscope from the colonies transferred. By direct observation with a low magnifying dry objective or an especial preparation-microscope, the colony to be transferred is picked out with a sterilized platinum needle. Some experience is required for obtaining the manual dexterity necessary to remove only the exact portions to be used for inoculation, and not to touch other portions of the surface with the needle before or after.

*b. Plate-Cultures.**—The glass plates necessary for use are of the thickness of a slide, and of such width that all points on their surface can be made accessible for microscopical observation. The relation of the width to the length depends upon the width of the stage of the microscope—about 8×14 or 10×12 cm. These plates are thoroughly cleaned (mineral acid, water, alcohol, ether), then placed in a tin box of corresponding size, covered over, and sterilized for two hours at a temperature of from 150° to 160° C. After cooling, such a glass plate is placed upon the glass plate of the apparatus (Fig. 16), and is protected from dust by a clean glass jar. The gelatin in a test-tube is then liquefied in a water-bath at 30° C., or by heating, and again so far cooled that it is somewhat consistent. The firmly fixed cotton stopper is then loosened by twisting with previously heated pincettes, so that its removal can be quickly and easily accomplished. It is advantageous to free the upper portions

* These were first demonstrated at the Hygienic Exhibition, and in an address by Koch at the Eleventh German Congress of Physicians at Berlin in 1883. More recently directions for these cultures have appeared from Biedert in an article in the "Deutsche Medicinal-Zeitung," 1884, and from Johne, "Ueber die Koch'schen Rein-Kulturen," 1885.

of the cotton plug from any possibly deposited germs by charring it in the flame. When the gelatin in the test-tube has been thus prepared, the tube is held with the thumb and forefinger of the left hand in a direction as oblique as possible, without the gelatin coming in contact with the cotton. Then the previously sterilized platinum needle or loop, while held by its glass rod in the right hand, is introduced into the material to be used for inoculation, thus removing a small portion; then the cotton plug is removed with the fourth and fifth fingers of the right hand, and the platinum needle or loop is passed into the liquefied gelatin, moved to and fro in it, wiped off on the sides of the test-tube, removed, and the cotton plug is again quickly replaced.

After this, the material introduced is mingled as equally as possible with the gelatin by turning, inclining, or gentle shaking; then the fluid gelatin, containing the germs thus separated, is poured out upon the cooled glass plate, and is spread out upon it with a glass rod or platinum wire, previously sterilized by heat and again cooled. This plate is laid upon a glass bench in a moist chamber, and over it a second glass plate is placed, so that here also several tiers of plates can be arranged in one jar.

While in the slide-cultures not the entire mass of gelatin, but only the lines of inoculation and their immediate surroundings are used, and while on this account a relatively large number of germs appear for separation along a relatively small line of inoculation, in the plate cultures, on the other hand, a relatively small number of germs are distributed in a proportionately much larger quantity of gelatin. Further, the division of the germs in the still fluid gelatin is far better, because the individual germs can develop into

colonies, being fixed in the solidifying gelatin and being isolated and more widely separated from other germs. An enormous number of germs can in this way be certainly separated from one another in a single plate-culture, in which each colony corresponds to a single isolated germ, and in which at first but few colonies are in contact.

All colonies which show differences are first examined microscopically, and then from the same, pure cultures are prepared by a second inoculation. For this purpose, in exactly the same manner, under the control of the microscope, a particle from a pure colony is introduced into new gelatin, and from this a new plate-culture is prepared, in which then, with the exception of possible air-infection, only this one organism develops. In these second inoculations, pure cultures can with certainty be obtained. Contamination by the air-germs can not be avoided during the opening, but it is not even approximately to be so much feared as infection by imperfectly sterilized instruments and hands, since these air-germs also develop in isolated colonies. If infection from the air has taken place during the opening and inoculation of the test-tubes, these air-germs can naturally develop in the same manner in the gelatin as those intentionally introduced; but their small number in comparison with the other organisms furnishes a suspicion of their source, and, moreover, not only a single plate-culture, but several should be made from the same material, so that one aids to control the other. If infection from the air has taken place after the solidification of the gelatin, this can be easily recognized by the position of the colonies on the surface.

In the larger number of cases this procedure suffices; but now and again in decomposing fluids, pus,

fæces, and very dirty water, the number of germs transferred with the drop or particle is so great that a sufficient isolation of the individual germs is impossible, because before the appearance of perceptibly characteristic differences in growth the colonies have come in contact with one another.

In these cases the dilution must be carried still further, and this can be done in two ways: first, the material taken by the platinum loop can be brought into sterilized distilled water, mingled with it by shaking, and from this greater or less diluted mixture a drop may be used for inoculating the fluid gelatin.

A second procedure is dependent on fractional cultures. A tube of sterilized fluid is first inoculated in the described manner, and is "the original." After thorough mixing, a few small drops—e. g., five—are in the same manner transferred from this tube to another for "the first dilution" (erste Verdünnung). The original tube is held between the thumb and index-finger of the left hand, and the tube to be inoculated between the index and middle finger; then the stopper from the original is removed with the pincettes and laid to one side, or it is placed between the fourth and fifth fingers of the left hand, which then holds simultaneously two tubes and the cotton plug. After this the plug from the second tube is removed by the fourth and fifth fingers of the right hand, the platinum loop is introduced into the original tube, and a small drop is transferred into the second. By a to-and-fro movement the drop is carefully removed from the loop, and then another drop is added, either with the same or a second platinum loop, previously prepared, and the same process is repeated about five times. Then the plug, held in the right hand, is placed in the newly inoculated

tube; afterward that in the left hand is placed in the original tube. Now the original (equals No. 0) and the first dilution (equals No. 1) are ready. Then a "second dilution" is made, and a third, in exactly the same manner, taking the same number of drops from the culture preceding. Each of these tubes furnishes a plate-culture designated by Nos. 0, 1, 2, and 3, which are placed in the same moist jar in tiers (Fig. 17). Each of these cultures controls the others.

If a number of different forms of bacteria have been separated, a plate-culture is made for each one of these, which, with the exception of possible air-infections, constitutes a pure culture of one of the single forms present in the original mixture. These inoculations of the pure cultivated organisms are repeated often, making peculiarly characteristic colonies, as tested microscopically, in order in this way to eliminate every soluble chemical constituent of the first substance.

c. Test-Tube Cultures.—On account of their slight protection, after standing a long time, it is scarcely possible to prevent the infection of the slide in plate-cultures by air-germs, which is especially inconvenient if a liquefaction of the gelatin is brought about by them. On this account it is necessary to renew the slide- and plate-cultures often, in order to avoid the accumulation of cultures occupying time and space, but especially to preserve certainly pure a culture once obtained. In order to do this, test-tube cultures are employed, in which, besides, many peculiarities of growth can be better noted.

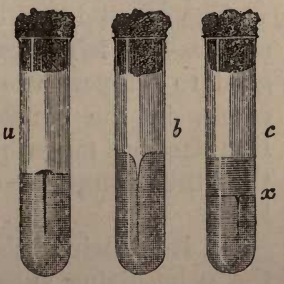
The test-tube, one third filled with solidified, sterilized nutrient gelatin, needs no other preparation than the loosening of the cotton stopper by turning with the heated pincettes in order to remove it quickly.

Then, with a sterilized platinum needle, a particle from the pure culture, under control of the microscope, is taken. The test-tube is held in the left hand so that the mouth looks downward; then with the fourth and fifth fingers of the right hand, or with the pincettes, the cotton plug is removed, and, with the platinum needle held by its glass rod between the thumb and index-finger, one or more thrusts are made into the gelatin (Fig. 18). Finally, while the mouth still looks downward, the cotton stopper is again securely replaced.

Fig. 18.

The changes in such a tube-culture after the inoculation with the bacteria vary considerably. As a general statement, the following data serve: Those forms of bacteria which do not liquefy the gelatin produce a characteristic surface-growth, extending out from the point of inoculation, so that soon a flat or more prominent head is formed, which, in connection with the line of inoculation of the culture, presents the form of a nail—"nail-cultures" ("Nagelculturen" of Friedländer) (Fig. 19, *a*).

Fig. 19.



In place of such a growth, others develop on the surface in the form of concentric rings, or present leaf- or grape-formed appearances. Some show an intense surface-growth and a want of development along the line of inoculation; in others this is exactly reversed. The cultures appear sometimes dry, sometimes mucoid; some are refracting, others transparent. The color of the cult-

ures is very variable. The gelatin is sometimes colored, sometimes not, and often a peculiar odor is produced. All of these small morphological and biological differences are to be noted, because they facilitate the differential diagnosis.

In the case of the bacteria that liquefy the gelatin, this is at times quite gradual, so that the gelatin-culture presents a delicate funnel-formed appearance (Fig. 19, *b*). In other cases it is more rapid; in these, wider funnels are produced or the liquefaction extends more in the form of layers. Sometimes a scum or mycoderma forms on the top; in others not. Often on the border-line between the liquefied and still solid gelatin (Fig. 19, *c*) the culture appears in the form of different-shaped clouds advancing forward. Sometimes the liquefaction seems closely united to the advances of the growth; many times it precedes it, as if the liquefied material was produced by the bacteria which extend farther than the visible growth reaches. In the case of the bacteria producing liquefaction also, the cultures may present different colors, and the gelatin may undergo change of color. An odor may be produced. These differences are collectively to be noted.

The adaptability of the ordinary gelatinizing substances—as isinglass, caraghen, and gelatin—in regard to temperature is limited to about 25° C., because, in the concentration to be used, complete liquefaction takes place at this temperature, and then the advantages of the solid nutrient media are lost.

If it is desired to make use of the advantages of the gelatinizing substances, transparency and solidity, at the temperature of the culture-oven, the vegetable gelatin called agar-agar, obtained from Graci-

laria lichenoides and Gigartina speciosa, is used in place of gelatin.

Instead of 5 or 10 per cent of gelatin, 1½ or at most 2 per cent of finely cut agar-agar is added to the solution. This is allowed to stand in an ice-chest for twenty-four hours to swell, and is then dissolved as completely as possible by boiling. The swelling can be accomplished in a shorter time by a slow heating. This hot solution is neutralized with carbonate of soda, and then cooked two hours on the water-bath, or one hour in the steam cylinder, for the separation of the substances precipitated by the neutralization and those coagulable by heat. Although the precipitated material may have been previously separated as much as possible by thick gauze, still these agar-agar solutions filter very badly, even in small quantities and in a hot-water filter.

According to Rosenbach, they are better filtered through cotton, while the glass funnel filled with cotton or glass-wool, and the vessel for receiving the filtrate, are placed in the steam apparatus. The filling of the tubes with the agar-agar solution and their sterilization is done in the same way as with gelatin. In thick layers the agar-agar seldom seems so clear as does a layer of gelatin of equal thickness. The inoculation of the plate-cultures is performed as usual; the solid agar-agar is liquefied at about 42° C. in a water-bath, and the inoculation and distribution of germs in the fluid-agar solution is accomplished while the tube is in part submerged in warm water, since these solutions under 40° C. lose their fluid state. At a temperature above 40° C., in which the liquefaction is more easily made, the germs are injured, so that the operation must be done between 40° and 42° C.

The pouring out of the inoculated fluid upon the plate, on this account, must be done as quickly as possible. A 1½ per cent solution of agar-agar-gelatin is so solid at 37° C. that all the advantages of the transparent and solid nutrient media are still preserved at this high temperature in the culture-oven. Many bacteria do not develop so well in agar-agar as in gelatin; but Rosenbach, however, has clearly shown the great superiority of this culture-medium, because most bacteria which liquefy gelatin, and which therefore produce no surface-growth, do not liquefy the agar-agar, but, on the contrary, show on it a very characteristic superficial growth.

The cultures upon agar-agar can be used to supplement the cultures in gelatin, and in this way the number of characteristics which are presented to the naked eye for differential diagnosis is materially increased. In the form of plate-cultures, with gelatin at the temperature of the room, with agar-agar for the culture-oven, Koch's method of the pure culture with the solid and transparent media affords, with the greatest simplicity, the highest guarantee for the certain separation of the different germs, however numerous, from a mixture of bacteria.

Improvised Means.

With the great simplicity already arrived at, on the purely technical side of the investigation, not much can be said in regard to improvised means, since also here complete certainty must be preserved under all conditions. The experiences, especially in hygiene, with the so-called expedited methods have taught all unprejudiced observers that only those who have worked out these methods, and those who are thoroughly experienced and practiced investi-

gators,' can use them sometimes with advantage and often without detriment, while beginners and inexperienced workers, for whom they are really intended, are, almost without exception, led by them into error.

All who would really study bacteriological investigation, or would found the important facts on a really practical basis, are by all means to be provided with a microscope having an oil-immersion system and an Abbé condenser, the most necessary apparatus. For a possible series of experiments on a more or less extensive expedition it is advisable not to take too much with you to perform this or that operation without the convenient aid of the laboratory instruments. Under these circumstances he only will improvise proper means who has had experience in laboratory work, and on this account can determine the limits to be reached with safety, or else these small improvised aids must be used under definite guidance as in other laboratory work; but then they have with improvisation nothing more to do.

In this sense, in place of the gas-flame, the flame of the spirit-lamp can be used. The culture-plates may be washed with a 1 per mille solution of sublimate and spirit, dried in the flame of the spirit-lamp, and sterilized in it by strongly heating the surface in its entire extent while the plate is held with pincettes. Then the plate, with the heated side up, is laid upon a clean piece of paper, which lies upon the table as horizontal as possible, and is covered with a soup-plate which has been cleaned with sublimate and spirit, and so allowed to cool.

If gelatin in test-tubes has not been taken, but a larger quantity of sterilized gelatin in a flask, test-tubes may be sterilized in the following manner: They are cleaned with a 1 per mille solution of subli-

mate and spirit; then the spirit is removed, and the tube dried by holding it over the flame of the spirit-lamp. A cotton plug is then shoved a few centimetres down into the tube with strongly heated pincettes. Now the lower portion of the tube is heated over the flame as far as the cotton plug. After the tube has so far cooled that it can be held by its lower portion, then the upper portion is heated so thoroughly and so long that the cotton is browned; when cooled, the plug is drawn so far out with the heated pincettes that it can be grasped with the fingers. A tube thus sterilized is filled one third full with gelatin, liquefied by placing in hot water; the plug is replaced, and then the gelatin is again carefully boiled, allowed to cool by dipping in cold water, and while it is yet fluid it is inoculated, and the germs thoroughly mingled with it.

The gelatin is not poured on the plate when it is perfectly fluid, because the plate does not lie completely horizontal; but it is done when the gelatin has become quite consistent, though before the appearance of lumps.

As moist chambers, two plates, laid one above the other, may be used, the lower one of which is covered with moistened filter-paper. Two pieces of wood laid upon this serve to support the culture-plate in place of the glass benches.

B. TRANSPARENT SOLID MEDIA, WITHOUT THE ADDITION OF GELATINIZING SUBSTANCES—BLOOD-SERUM.

For some pathogenic bacteria the transparent solid media, in the form of gelatin and agar-agar cultures, are not suitable. For these cases Koch devised, while he fully preserved the principle of the solidity and transparency of the culture-medium, a quite

different way. Koch* had observed that sterilized blood-serum, when heated above 65° C., but before reaching coagulation temperature, became solid without losing its transparency. Such solid, but transparent, blood-serum was then introduced as a nutrient medium.

For collection of the blood, cylindrical vessels provided with glass stoppers, about 29 cm. high and about 8 to 10 cm. wide, are used. These vessels, after previous mechanical cleaning, are sterilized by washing with 1 per mille sublimate. After pouring away the sublimate, the remainder of it is removed with alcohol; the alcohol is poured off and the traces of it removed with ether, and this last is evaporated by warming in the dry-oven. After removing the glass stopper, the blood of the slaughtered animal is allowed to run into this vessel. The neighborhood of the cut opening must be well cleaned, or, at least, must be thoroughly moistened. The blood flowing out immediately after the cut, which washes away the dirty particles of the skin, the fur, and the cut hair, is not preserved.

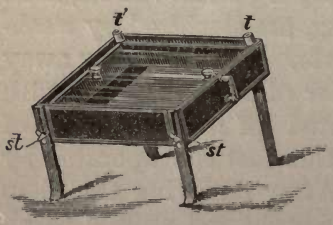
The vessel is now filled to the brim, closed with the stopper, and, as soon as possible, placed in an ice-chest, in which it remains standing from twenty-four to thirty hours, in order to render possible the formation of a solid blood-clot. If the vessel is moved during the formation of the clot, the blood-corpuscles are mingled with the serum, which prevents the latter from becoming completely clear after warming. After the specified rest and time a rich layer of completely transparent, amber-colored serum forms above the blood-clot. This is removed with a

* "Berliner klinische Wochenschrift," 1882, No. 15, und "Mittheilungen aus dem kaiserlichen Gesundheitsamte," II, 1884, S. 48.

sterilized pipette and transferred to sterilized test-tubes, which are filled one third full, and then closed with cotton plugs.

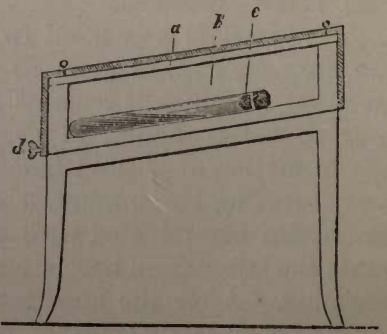
Sterilization can only be accomplished below the coagulation temperature of albumen by discontinuous heating. For this purpose the test-tubes are placed in the apparatus (Fig. 2). The temperature of the inner room (*b*) is kept at about 58° C. The blood-serum is exposed to this temperature on five or six successive days for about one hour daily. This can also be accomplished by means of a water-bath, but less conveniently. Upon the fluid, sterilized blood-serum there often forms a scum of cholesterin, which should not be confounded with the mycoderma produced by bacteria.

Fig. 20.



This sterilized, fluid blood-serum, which is often used as such, is placed, for bringing about solidification, with the test-tubes inclined at a sharp angle, for attaining the greatest possible extent of surface. For this purpose a double-walled box, made of zinc, provided with a glass cover, is used. The chamber between the double walls is filled with water. The front side of this box can be lowered by means of set-screws (*st* in Fig. 20, *d* in Fig. 21). The box is then

Fig. 21.



placed at such an angle that the blood-serum reaches to the upper third of the test-tube; but care should be taken that it does not come in contact with the cotton plug.

The regulation of the temperature of the air-chamber (*b*, Fig. 21) is accomplished by a thermometer placed between the test-tubes upon the floor. Solidification takes place at 65° C.; the higher the temperature rises above 65° C., and the more it approaches the coagulation temperature of 75° C., the more rapidly solidification occurs; but transparency is more surely obtained the lower the temperature is used. The serum always becomes opaque when the temperature approaches the point of coagulation. On this account it is best to keep the temperature as nearly as possible at 65° C., and at least not to allow it to rise above 68° C. The blood of different animals solidifies with different rapidity—the blood of sheep most rapidly, calves' blood most slowly. Generally, solidification takes place in from one half to one hour's time.

Blood-serum that has been properly solidified is as solid and firm as hard-cooked white of egg, similar to amber, transparent, and only in the lower third a light milky cloud appears.

During the heating, water of condensation formed on the upper wall of the test-tube, which is coolest, collects at the bottom of the test-tube when it is placed upright, and forms, by absorption of soluble substances, a nutritive solution, so that the simultaneous growth upon solid and fluid nutrient media may be observed after the inoculation, if the inoculation extends to the edge of the fluid.

By evaporation the serum gradually dries, beginning from above, yet the middle and lower portions

remain available for months. Löffler showed that solutions which heighten the nutrient value of the blood-serum, when added in small amounts, do not diminish its capacity for solidifying in a transparent form, so that in place of the pure blood an improved blood-serum can often be advantageously used. Löffler * adds to three parts of blood-serum one part of beef-infusion. The beef-infusion is prepared exactly after the manner of Löffler's gelatin (page 133); 1 per cent of peptone, 1 per cent of grape-sugar, and ½ per cent of chloride of soda is added; the solution is boiled, neutralized with carbonate of soda, boiled again on the water-bath until the albumen is completely precipitated, and then filtered. This bouillon is sterilized in the steam-cylinder, and, after cooling, is added to the serum; and then the serum, with the added bouillon, is sterilized by discontinuous heating, and solidification is brought about. It is self-evident that other substances may also be added—e. g., solutions of beef-extract with sugar, etc. Since the test-tube cultures can not be directly observed with the microscope, blood-serum may also be allowed to solidify in watch-glasses, or in hollowed-out glass blocks which are covered with glass plates; but then the greater protection which the cotton stopper of the test-tube affords is lost.

For inoculation of the test-tubes, they are held in the left hand as nearly horizontal as possible; then a platinum loop is dipped into the substance to be inoculated, the previously loosened cotton plug is drawn out with the fourth and fifth fingers of the right hand, and the germs are transferred to the surface of the solidified serum by firmly drawing lines with the

* "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. II, 1884, S. 452 und 461.

platinum loop upon the same. The cotton stopper is then again replaced.

Since in test-tubes, on the one hand, a direct microscopical control, and on the other an isolation of the germs in the solidification, is not possible as in gelatinizing substances on slide- and especially plate-cultures, many individual experiments (say ten) must be made with each substance, and, aside from this, the cleanest possible inoculating material must be obtained. These two facts make the blood-serum cultures, as contrasted with gelatin and agar-agar cultures, seem incomplete. But, by overcoming this technical difficulty, it is possible, on account of the retention of the principle of transparency and solidity, which distinguishes Koch's method so fundamentally from all others, to obtain on solid blood-serum faultless clean cultures of obligatory parasitic bacteria, which can not be obtained by any other method.

The method of obtaining pure original material for the blood-serum cultures needs still some explanation, especially for the cases in which, as in tuberculosis, the looked-for organism develops so slowly that, in the interval, contingent rivals outnumber it.

In the preceding microscopical examination of sections of different organs of tissue-fluids and blood, it is to be determined at what places the suspected bacteria are to be found most certainly, and in the purest condition. After observation of these points, it is shown that the removal of the inoculation-material is best performed when it is taken from an animal recently dead or killed.

The removal is proportionately more difficult and uncertain the longer the time that has elapsed after death, because then the possibility of a mixture with the rapidly developing micro-organisms of decomposi-

tion is always greatly increased. "All preparation cuts, which do not come in contact with the inoculation substance, are to be made, according to Koch,* with hot instruments, but the inoculation mass itself is removed with cooled scissors and pincettes, or with the cooled platinum loop. The operation should be always done with heated instruments, which should be changed every time when a new section is to be laid open. This constant change of the instruments is necessary, that the contamination which adheres to them in cutting through the skin and the superficial layers may not be introduced into the cultures."

With regard to the previous preparation, it should be noted that, after placing the animal upon the dissecting-table, the skin, so far as the cut is to extend, should be thoroughly moistened throughout its extent with a 1 per mille solution of sublimate, in order to prevent as much as possible the scattering of dust, hair, etc., in seizing and cutting.

A number of knives, scissors, and pincettes are previously heated in the flame, and laid under a jar to protect them against contact and dust. Then the skin along the line for the incision—in the larger animals with a hot scalpel, in the smaller with hot pincettes and scissors—is cut through and laid back on both sides, so far that further operations will be unimpeded. With a second pair of hot pincettes and scissors, if it is desired to remove the pleura or surface of the lung, an opening is made in the wall of the thorax, 1 by 2 cm., and thus the surface of the lung is laid bare. If in this way an infected portion is exposed, for instance, nodules of tubercles, one or more of these nodules is snipped out with the cooled instruments. In order to release the bacilli found in

* "Mittheilungen," Bd. II, 1884.

the tubercle nodules, a nodule is cut with a cooled knife or scissors, and a particle from the interior is removed with the cooled platinum loop and transferred to the surface of the blood-serum; or a nodule is crushed between two cooled scalpels, and this crushed mass is taken with a platinum needle and transferred to the blood-serum. If it is less consistent, it is cut into with the cooled knife and the material removed with a platinum loop. If blood is to be taken from the heart, after the skin has been cut in the same way, the wall of the thorax over the heart is opened with hot pincettes and hot knife or scissors: then, in the same way, the pericardium is opened with fresh instruments. After this the apex of the heart is fixed with cooled pincettes and a cavity opened with a scalpel, out of which the blood to be used for inoculation is withdrawn on a platinum loop.

If an organ from the abdomen is chosen, this is removed with heated instruments after the abdominal cavity has been laid open in the same manner, and is placed upon a clean dish or filter-paper, and then only the connective-tissue capsule is cut into. After this the edges are seized with cooled pincettes and a deep tear made in the organ; then with a platinum needle, out of the bottom of the rent, the tissue, fluid, or particles are removed.

For the superficial lymph-glands the skin is cut through in the same way; then the gland is fixed with cooled instruments, and is opened *in situ* for removal of material from the interior, or it is released from its surroundings, laid upon a glass plate previously heated and cooled, cut or torn, and then fluid or a particle of tissue is taken from the interior.

If blood is to be taken *intra vitam* for cultures, this can be done with the capillary-tube of Salomon-

sen; also, after opening a vessel, a drop of blood can be taken from the interior with a platinum loop. It is simplest to free a spot from hair, clean this with brush and soap, wash it with a 1 per mille solution of sublimate, then with alcohol, and finally remove the last particle of sublimate with ether, after which a sterilized needle is introduced or a small cut is made with a sterilized knife. The first few drops of blood welling forth are removed with a platinum needle, and those coming later are used for inoculation.

For removal of a piece of the skin in erysipelas and similar parasitic processes extending in the skin, a spot is chosen in which the process is advancing. The skin is cleaned in the same manner, and then, with heated instruments, a piece is snipped out which finally is still further reduced in size or crushed.

If some time has already elapsed since death, the organs must be thoroughly cleaned from the organisms of putrefaction adhering to the exterior. According to Koch (*l. c.*), this is accomplished by repeatedly and thoroughly washing the organ in a 1 per mille solution of sublimate. Then with hot instruments, changed at each cut, sections are removed from the surface of the organ, and only at a greater depth the tissue-fluid or particle is taken.

In the larger organs—e. g., the spleen—according to Gaffky,* after thorough washing in a 1 per mille sublimate solution, a long cut is made, almost dividing the entire organ; then, with a second sterilized knife upon the clean-cut surface, a section is made that does not extend at any point to the capsule. Upon this a third is made, and then out of the depth tissue-fluid or a particle is taken.

* "Mittheilungen aus dem kaiserlichen Gesundheitsamte," 1884, Bd. II, S. 386.

According to Löffler,* in order to make even the very unclean organs useful, the organ is washed for ten minutes, with a continuous motion by a glass rod, in a 5 per cent solution of carbolic acid in order to destroy all the cocci, bacilli, and fungi adhering to the surface. For the destruction of contingent spores of bacilli, the organ is placed for five minutes in a 1 per mille sublimate solution, and laid upon clean filter-paper. After the surface has become dry, the connective-tissue capsule is cut into with a hot knife, the organ is torn by seizing the edges of the cut with hot pincettes, and out of the bottom of this rent inoculation-material is taken.

The course of the culture ought to be the same as in individual cases which follow from the spontaneous presence of bacteria.

In the case of the septic forms, and the pigment and ferment bacteria, the object is always well attained with plate-cultures of gelatin or agar-agar. Hereby the small advantages which a quantity-culture possesses are most favorably employed, especially with regard to the heating and anaërobiosis which may be realized in the plate-cultures also.

Among the parasitic bacteria there are, with the greatest probability, some as to which epidemiology furnishes evidence that they are probably optional parasites or optional septic forms, and can live entirely, or, under certain conditions, outside of the animal organism. These can be obtained pure in gelatin or agar-agar cultures.

For the obligatory parasitic bacteria—as are, in a greater or less degree, the parasites of the purely contagious diseases—only the blood-serum cultures,

* *Ibid.*, S. 451.

up to the present time, offer the possibility of obtaining pure original material.

For those bacteria, as spirilla and spirochætæ, which as yet it has been impossible to cultivate upon solid nutrient media, or only badly, and for those more movable parasitic micro-organisms, which possibly may be the cause of one or another disease, it is best to recur to the tested method of dilution—the "one-cell culture." This method is exceedingly minute and not quite so reliable, but surpasses all others and is far preferable, if other means can be previously used in order to secure, if not absolutely, yet approximately, pure quantity-cultures.

For other micro-organisms, as mould-fungi and yeast, the solid nutrient media, both in the opaque form of layers of potato, of potato-broth, and a thick bread-broth, are of value, and also the transparent forms of gelatin, agar-agar, and blood-serum cultures. Attention must also be given to the reaction, which, as a rule, contrary to the cultures of bacteria, should be kept feebly acid; and also to the consideration of the spontaneous presence of the germs on the nutrient media.

While previously the pure cultures had only been considered a means intended for unobjectionable positive inoculation, Koch has shown, upon the ground of the biological peculiarities manifested in the pure culture, that there was not the slightest reason to believe that the ætiology of anthrax is explained by the gradual adaptation of a quite harmless bacterium to a parasitic mode of life, but that the anthrax bacilli are not at all dependent on the animal body for the retention of their species, and that they find, outside of the organism, at least for a time, all the condi-

tions necessary for their existence, and that their parasitic existence is only occasional.*

Then Koch succeeded for the first time in cultivating, artificially outside of the animal body, the parasite of a purely infectious disease, tuberculosis,† and Brefeld ‡ showed, on the ground of his investigations regarding the septic stage of the parasitic fungus of the cereals, that it was also possible, by the use of the right methods, to bring out from its parasitic life this germ—a parasite in the narrowest sense.

In conclusion to this, I suggested # that each pure culture is nothing else than the septic stage of a parasitic micro-organism; and likewise de Bary ‖ perceived the capacity of parasitic bacteria of being bred in dead organic material.

* "Zur Etiologie des Milzbrandes." "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. I, 1881, S. 49.

† "Berliner klin. Wochenschrift," 1882, No. 15.

‡ "Die künstliche Kultur parasitischer Pilze." "Botanische Untersuchungen über Hefenpilze," V, 1883, S. 1.

"Fortschritte der Medizin," 1884, No. 2, S. 70.

‖ "Vergleichende Morphologie und Biologie der Pilze," 1884, S. 526.

IV.

INOCULATIONS FOR THE DETERMINATION OF THE CAUSAL RELATION OF BACTERIA-GROWTH TO DECOMPOSITION AND DISEASE.

A. Septic Bacteria.

Substances capable of decomposition, such as solutions of sugar, glycerin, etc., are prepared according to the indications which the spontaneous presence of bacteria suggest. The details of the preparation have been already mentioned in the description of cultures in fluids, as also the methods of sterilization. For the qualitative experiments, test-tubes and small flasks are filled with the solutions; for the quantitative experiments, flasks containing a litre and still larger are used, which have applied, over the cotton stopper, caps made of a double layer of filter-paper. If it is desired to investigate what degree of decomposition the bacteria spontaneously bring forth, then the solutions receive no additions; but if the substances are to be chemically changed, a corresponding quantity of the purest carbonate of lime is added, in order to neutralize the acid formed in proportion to their development.

Inoculations are made by picking out, with a platinum needle, under control of the microscope, a particle from a pure culture in gelatin or agar-agar, and introducing it quickly into the solution. In these

cases not only one germ, but a great number of the same germs are introduced, which in this way are immediately transferred into a position to more easily suppress contingent rivals in the favorable solution. If the pure culture was obtained by the method of dilution, as a one-celled culture, then the unit of volume (a drop, a cubic centimetre) with the hypothetical germ is transferred, by means of a sterilized pipette, to the sterilized solution.

Since air-infection in a fluid is not immediately recognizable, and the possibility referred to is never entirely excluded from cultures in fluids and fractional cultures, and since in some way such an accidental germ introduced from the ordinary septic bacteria may outgrow all others, a greater number of individual experiments must be made in all investigations with fluids. It is recommended that these transfers be made in a glass case kept moist.

An elevation of temperature favors the growth and action of all septic bacteria, but the temperature most favorable to the different forms is limited, and for many ferment bacteria it is about the temperature of the blood. On this account, after the inoculation of the solutions, some must be kept at the temperature of the room and others at a higher temperature in the culture-oven, in order to approximately determine the favorable temperature in a short time.

Control is followed out in three directions: first, by exposure of sterilized, uninoculated solutions to the same external conditions; secondly, by the use of the microscope; and, thirdly, by pure cultures. If in the inoculated solutions a change becomes visible to the eye by a cloudiness, by formation of a mycoderma, or by the appearance of air-bubbles, the

solution is tested microscopically by a dried cover-glass preparation.

The drop of fluid must be carefully taken by sterilized instruments, a platinum loop or pipette. Care should be taken to see whether only one and the same form is present, and whether the form is identical with that observed in the pure culture. Small differences may appear, because the solutions are more favorable to the bacteria than nutrient gelatin. Then, at the height of the fermentation, and more often toward the end, sometimes involution-forms appear, or there occur, in a more typical manner, in the individual bacteria, especially in the bacilli, peculiar enlargements, which produce in the rods whetstone, spindle, or tadpole forms. If such appearances are observed, it is to be determined whether these forms are associated with the activity of the fermentation—i. e., heightened function—or whether, on the contrary, they constitute the first visible indications of the total or partial exhaustion of the nutrient medium ; or whether they prepare for the formation of spores, by which, in the exhaustion of the medium, the preservation of the species is rendered sure.

Concerning this the microscope alone gives no information ; consequently, if these forms are seen, cultures are made, in which different species develop, if in the inoculation foreign bacteria have been included ; but only the inoculated forms develop after a really pure transfer.

With the determination of the most advantageous temperature, the quantity of oxygen most favorable to the development is also to be especially observed. While many septic bacteria—viz., those producing oxidation decomposition and the ordinary aërobic

forms—present no especial peculiarity in respect to this, and, with the admission of air both in fluids and in gelatinized solutions, can be easily obtained pure, other forms show a greater intensity of growth and action if the entrance of air is not quite free. When they appear spontaneously these bacteria have a tendency to hydrobiosis—i. e., they develop less on the surface and more in the interior of the fluid. In these cases the entrance of oxygen should be restricted; but the oxygen in solution is always at the command of these bacteria. It is very doubtful whether the extreme ground is tenable which Pasteur takes for the departure of his theoretical observations on decomposition—viz., that the anaërobic forms are so constituted that the air acts as a poison, since in all these experiments still other factors come into play.

Also, the anaërobic bacteria can be obtained pure by gelatin and agar-agar cultures, because, in most cases, a restriction of the entrance of air guarantees a sufficient intensity of the development. This can be better attained in the thick layer in the test-tube cultures, and still better if, according to Koch,* upon the gelatin or agar-agar plates a thin sheet of isinglass or mica is laid which covers at least a third of the surface of the gelatin in the center. This should be applied as the gelatin begins to solidify. "The mica-sheet, on account of its elasticity, adapts itself completely to the surface of the gelatin and excludes the air from the portion covered." Anaërobic bacteria develop into colonies under the mica-plate, while exquisite aërobic forms grow well only about 2 mm. within the edge of the plate, "so far

* "Conferenz zur Erörterung der Cholerafrage." "Berl. klin. Wochenschrift," 1884, No. 31, ff. Und "Deutsche med. Wochenschrift," 1884, No. 32, ff.

as a diffusion of the air can take place." Of the aërobic bacteria only extraordinarily small colonies, not visible to the naked eye, are formed under the mica-plate. These probably prolong their existence by means of the oxygen contained in the gelatin, but can not subsequently develop farther. The inoculated plate- and test-tube cultures without these mica-plates can be placed under the jar of the air-pump, or in a jar in which the air has been replaced as much as possible by carbonic acid.

In this manner it is possible to make available the advantages of the solid and transparent nutrient media for obtaining pure cultures also of the anaërobic bacteria.

Experiments concerning Anaërobiosis in Fluids.—It is necessary further to make experiments concerning anaërobiosis in fluids in order, on the one hand, to study the decomposition with the absence of air or restriction of its admission; and on the other, also, to separate, by the aid of anaërobiosis in measure-cultures, the anaërobic bacteria from the purely aërobic—as was done by Fitz,* with the result of winning, by dilution from this original material, a one-cell culture.

The experiments concerning anaërobiosis may be divided into two classes: (*a*) those in which only the air is expelled, and (*b*) those in which the air is replaced by other gases. The expulsion of the air can be accomplished by boiling, and then it must be possible to make the inoculation after cooling, so that in the inoculation no air can gain admission. Either the inoculation of the solution with a pure culture in gelatin, or a one-celled culture, takes place before

* "Ueber Spaltpilzgährungen," IX. "Berichte der deutschen chemischen Gesellschaft," Bd. XVII, 1884, S. 1188.

the expulsion of the air, and then the air must be driven out at a temperature which can not be injurious to the bacteria.

It is advantageous in the first case, necessary in the second, that the solution to be inoculated be previously thoroughly sterilized.

Fig. 22.

The simplest order of experiments for examination and for obtaining quantity-cultures consists in filling a flask (*a*, Fig. 22), having a long neck, half full with the impure mixture of bacteria which is to be tested as to the presence of anaërobic bacteria, or with the sterilized, inoculated solution; then the neck is drawn out narrow in the gas-flame at *b*, and the open end (*c*) is joined to an aspirator or air-pump. During the exhaustion of the air the flask (*a*) is placed in a water-bath at about 38° or 40° C., in which it remains boiling strongly for about half an hour; then the neck at *b* is sealed in a pointed flame, while the suction is continued.

Fig. 23.

In this way a water-hammer quite free from air is obtained, which strikes strongly upon the wall, and in which the fluid boils at the temperature of the body. According to the material, these flasks are kept at the temperature of the room, or in a culture-oven.

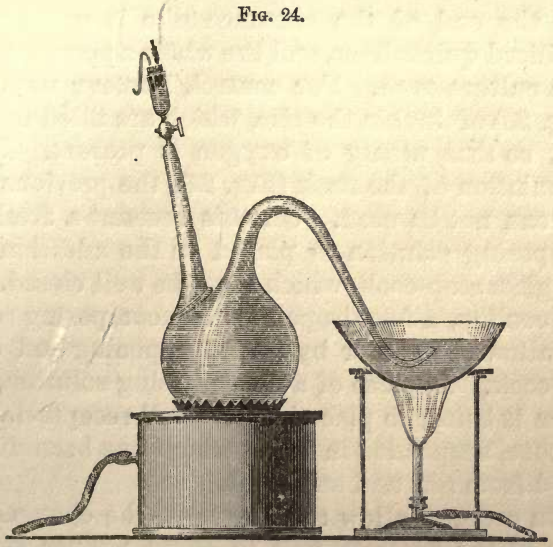
Nencki* proceeds in the following manner (Fig. 23): The vessel is filled half full with

* "Beiträge zur Biologie der Spaltpilze," 1880.

the mixture of bacteria or the inoculated solution, and afterward closed with the double-perforated rubber stopper. Through one opening of this stopper a glass rod (*b*) passes, which ends in a ground stopper that fits closely in the constriction at *c*, so that the contents of the ball (*A*) are completely shut off from the fluid above. In the second opening a tube (*d*), bent at a right angle, is placed, the end of which is connected with an air-pump. During the suction the ball (*A*) is placed in the water-bath, and the glass rod (*b*) is withdrawn so that the stopper rests at about *a*. As soon as the air is removed, which is indicated by the shocks produced by the boiling and striking of the fluid on the walls of the vessel, the glass rod is pressed downward by careful turning, until the fluid in the ball (*A*) is completely cut off by the stopper. Then, during the suction, the tube is sealed at *d* in a pointed flame, and, after the cooling of the sealed end, is broken in a concentrated, recently prepared alkaline pyrogallic solution. After this solution has accumulated until it reaches about the height of *n* the tube is sealed again at *d*. The alkaline pyrogallic solution serves as an index for the surety of the closure, as the air, gaining entrance, would change the clear yellow-brown solution into a dark color. In these experiments no precaution is taken against the escape of the gas. Pasteur* endeavored to obtain this in the following manner (Figs. 24 and 25): A flask, of the form shown in Fig. 24, is filled with the solution; the tube leading into it is submerged in a porcelain vessel filled with the same solution. The fluid in the flask and that in the porcelain dish are boiled simultaneously for about half an hour, in order

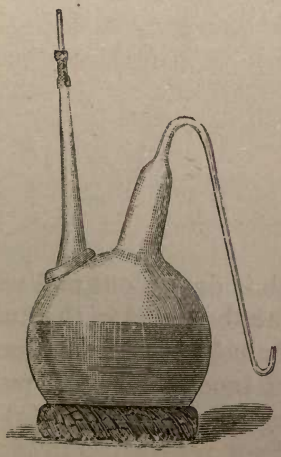
* "Études sur la bière," 1876, S. 229, ff. "Des rapports de l'oxygène avec la levure."

Fig. 24.



to expel all the air or the oxygen of the air out of the fluid. By the development of steam the fluid is driven out of the balloon, but is replaced again by the fluid in the porcelain dish, which has been rendered free from air by boiling. This repeats itself many times during the boiling process, and it is then allowed to cool. During the cooling I have found it advantageous to cover the fluid in the porcelain dish with a layer of oil, previously sterilized by boiling an hour, in order to prevent, as much as possible, the subsequent absorption of oxygen. After complete cool-

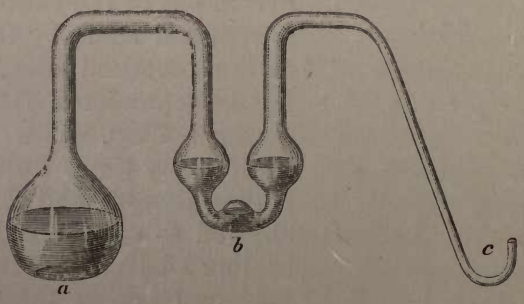
Fig 25.



ing, the end of the entrance-tube is submerged in sterilized quicksilver, and the whole apparatus placed in a culture-oven. For control, Pasteur uses flasks (Fig. 25) of double the size, which are filled only half full, so that plenty of oxygen is present. For the inoculation of the flask (Fig. 24), the previous methods can not be used. On this account a freshly decomposing solution is placed in the addition above the glass stop-cock, which must be well closed. After the cooling, a few drops of the decomposing solution are allowed to enter by quickly opening and closing the cock. In place of a decomposing solution, I consider it better to place in the small receptacle a pure culture, suspended in water which has been distilled, freed from air, and sterilized.

In order to allow the escape of the changed products of the bacteria, and to avoid rubber stoppers, Nencki chose the following apparatus (Fig. 26, *l. c.*):

Fig. 26.



The entrance-tube (*c*), which is drawn out and narrowed at three or four places, is submerged in the fluid to be tested for the presence of anaërobic micro-organisms, or in the sterilized fluid inoculated with pure cultures, while at the same time the litre-flask (*a*) is freed from air by heating. As a result, the fluid is

aspirated into the apparatus until about two thirds of the flask (*a*) is filled.

Then the end (*c*) is connected with an air-pump, and, while the flask (*a*) is placed in a water-bath, the entire apparatus is exhausted of air, as previously described (text for Figs. 22 and 23). During the suction the tube (*c*) is constricted and sealed. The sealed end is then placed in pure carbonic-acid gas or nitrogen and broken, and thus the apparatus is filled with this gas and the end (*c*) is sealed anew. The sealed end is then again broken in an alkaline, concentrated pyrogallic solution, and, by heating the apparatus, a portion of the gas is expelled until the two balls of the U-formed tube (*b*) are about half filled with the pyrogallic solution. Then the end (*c*) is submerged in quicksilver and the entire apparatus placed in a thermostat.

Gunning,* doubting the value of the previously described arrangement of experiments, has claimed that the protections used against the admission of air—such as stop-cocks, quicksilver, and pyrogallic solutions—offer no absolute certainty, since he was able to determine the presence of oxygen, as he believed, by that reagent which he thinks most delicate for detecting oxygen—viz., double ferro-cyanide ($Fe_2[FeCy_6]$).

Nencki and Lachowicz† chose, on this account, the following arrangement for experiments (Fig. 27): the apparatus consists of two flasks (*A* and *C*), each containing about 250 c. cm., and one flask (*B*) containing about 50 c. cm.

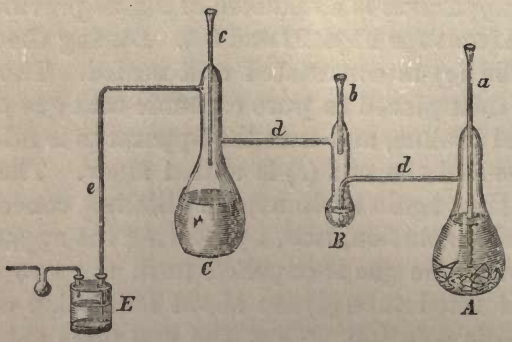
The flask *A* serves for the development of hydro-

* "Journal f. pract. Chemie," N. F., Bd. XX, 1879, S. 454.

† "Die Anaërobiosifrage." "Archiv f. d. ges. Physiologie," Bd. XXXIII, 1883.

gen from iron and sulphuric acid, and for the formation of sulphate of iron free from the oxide. On this account, before the sealing in of the funnel-tube (*a*),

FIG. 27.



a number of rolls of piano-wire are placed in *A*. *B* is used for the reception of about 10 c. cm. of a solution of prussiate of potash, and for the development of the double ferro-cyanide of iron. *C* contains the nutrient solution. The flasks are carefully cleaned, or, better, sterilized and dried by heat, and then at *d* joined together. After this, *A* is filled two thirds full with distilled water, rendered acid by a few drops of concentrated sulphuric acid, and heated to boiling. During the boiling care should be taken that the steam condensed in the tube (*d*) between *A* and *B* flows back into *A*, by placing the apparatus in an inclined position, because particles of the oxide of iron may be drawn into the flask *B* with the steam. "After the water has boiled for a few minutes, it is allowed to cool to 50° or 60° C., and about 30 to 40 per cent of sulphuric acid, well boiled, is poured through the funnel-tube (*a*) from time to time in small portions, by which an equal and rapid stream of hydrogen is produced that lasts many hours. Now a few fresh

crystals of ferro-cyanide of potash are dissolved in thoroughly boiled water, and a few c. cm. are poured from this through the funnel-tube into the flask *B*. While the oxygen passes through the ferro-cyanide of potash solution for about fifteen minutes, the flask *C* is filled about two thirds full with the sterilized nutrient solution, and then the flask *B* is sealed at *b*. Now the fluid in the flask *C* is kept at the boiling-point for ten or fifteen minutes; after this the flame is removed, and, while the fluid is still boiling, the entrance-tube (*e*) is submerged in the quicksilver (*E*)." The quicksilver is covered with a layer of alkaline-pyrogallic solution, and this with a layer of oil or some other fluid insoluble in water. The funnel-shaped tube (*c*) is now closed with a wax stopper, and then the hydrogen gas escapes through the quicksilver closure at *E*, and thus expels all the air from this portion of the apparatus. The wax stopper is removed when the nutrient solution in *C* is cooled to about 30° C., and it is then inoculated with a few drops of the mixture of bacteria to be tested, or of the pure culture suspended in sterilized water. The neck at *c* is dried with filter-paper and sealed. In order to fill the tube (*a*) with hydrogen, some iron wire is likewise placed in it, and this tube (at *a*) is also sealed. Now, in the entire apparatus the air has been displaced by hydrogen. "In this manner, throughout the entire extent of the apparatus, is a stream of hydrogen passed, and the development of the gas continues for some hours after all the entrance-tubes are sealed. By inclination of the apparatus some of the solution of oxide of iron is poured out from *A* into *B*; the resulting precipitate of the double ferro-cyanide of iron is completely white, and does not alter its color even after long standing."

Since the question of anaërobiosis is by no means settled in all respects, the direction of the investigation may be guided by the fact that while Pasteur, Nencki, and most investigators accept anaërobiosis as proved, Gunning denies the same, and designates it as a phantasy. In the experiments, for this reason, it is especially necessary that a simple hydrobiosis should not be accepted as anaërobiosis, but that special experiments be undertaken for this.

If anaërobiosis is recognized, it is to be determined whether this is the cause of the decomposition (Pasteur), or whether it has a more insignificant relation to the course of the decomposition. As for myself,* the entire question seems to me to depend upon the recognition of the possibility of anaërobiosis.

B. Parasitic Bacteria.

By the inoculation of susceptible animals with pure cultures (*vide* Infection-Methods, page 125) it is directly shown that micro-organisms present in a diseased process are really the *conditio sine qua non* of the respective infectious disease. The indirect methods for the investigation of the ætiology, also the experimental investigations acting locally, all tend only to this point. A restriction is often placed here to the methods. In studying the statistics, also large in numbers, we must here halt, and consider the fact that in the valuation of such great numbers the value of the conclusion is very dependent upon how the statistics have been collected, since the same material has been brought forward repeatedly with the

* "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. II, 1884, S. 345; und "Ueber die Zersetzungen der Milch und die biologischen Grundlagen der Gährungsphysiologie." "Deutsche med. Wochenschrift," 1883, Nos. 48 bis 50.

same resoluteness, sometimes to support the soil-water theory of disease, sometimes the drinking-water theory, sometimes for the explanation of disease as of a contagious and sometimes as of a miasmatic nature.

It would be a sad error to underestimate the significance of the ætiological investigation directed to the study of the accessory causes of disease. This investigation often furnishes valuable points for practical treatment by the denial of other facts, but it is also wrong to ascribe to the results of this investigation the only value, and to regard the experiments on animals as showing nothing concerning the natural mode of infection. It would be well if one now and then recalled the often serious defects of the methods with which the indirect investigation into ætiology must be conducted, and in which the certainty of the conclusions drawn are often sharply contrasted with the uncertainty of the material from which the observations are made.

But we must also avoid prejudice in the experiments on animals. The experimenter who, possessing susceptible animals, completely masters the experiments, easily falls into the error of believing too strongly in contagion, and of concluding too much from his direct results as to the natural method of infection.

The problem in the experiments on animals is in reality a twofold one, but in the division of which, nevertheless, a clear dividing-line can scarcely be drawn. In the first place, it is to be determined by direct inoculation experiments whether a form of bacteria is pathogenic or not, and whether it is the cause of a disease or a possible accompaniment. In these experiments it is at the same time to be determined whether the bacteria, after the artificial in-

fection, are distributed and arranged in the same way as when the disease appears spontaneously in the body, so as to explain clearly all clinical symptoms and pathological lesions. Secondly, we should endeavor as nearly as possible to determine the method of infection which, from the clinical, anatomico-pathological, and general epidemiological observations, is shown to be the method of the natural infection in the spontaneous appearance of an epidemic disease. The following methods for infection can be used:

Inhalation Experiments.—The inhalation experiments are performed by placing the animals in a spacious box, the air of which is mingled with organisms. This is done by means of a steam-dispersing apparatus, or, less conveniently, by a hand-spray. Either the apparatus is allowed to act for a short time—twenty minutes to an hour—and in this case pure cultures, suspended in large numbers in sterilized water, are used, or the dispersion is continued for a longer time, and then the bacteria are mingled in a correspondingly larger mass of water. Since, in natural infection by the breath, spores probably play an important *rôle*, this is to be taken into consideration in the experiments, and many of the differences concerning the negative or positive results in the production of tuberculosis by inhalation of tubercular sputum can be traced back to this. According to Veraguth,* 10 grammes of purulent tubercular sputum are rubbed up with 500 grammes of water. This mixture is filtered—to remove the accompanying material held in suspension, which might produce an inflammation of the lung-tissue by its mechanical or chemical action—through two thick layers of flannel,

* "Experimentelle Untersuchungen über Inhalations-Tuberculose." "Arch. f. experim. Pathologie," 1883, Bd. 17.

or, according to Weichselbaum,* through absorbent cotton.

In inhalations through a tracheal fistula, precautions should be taken to avoid the possible simultaneous infection from the wound. If it is desired to see what action bacteria produce when they are introduced into the trachea in any way, according to Küssner,† the trachea may be laid bare by a small cut, made with antiseptic precautions, and the material introduced through a fine opening into it by means of a syringe.

Reception of Bacteria with the Food.—Feeding Experiments.—In the artificial infection by feeding, the possible injury of the mucous membrane which may be produced by rough, prickly food, must be avoided. Infection through such an eroded spot is to be viewed as a wound-infection in its entire effects, and regarded as having a different significance, as to the ætiology of the spontaneous appearance of the disease, from the reception of infectious material through the food without erosion of the mucous membrane.

According to Koch, Gaffky, and Löffler,‡ this object is most certainly attained, at least in the larger animals, in the following manner: a thin layer is so far cut from a small, fresh rectangular piece of potato that it can be lifted up like a cover, and then the interior is hollowed out and filled with the bacteria-material in pure culture. The cover is replaced, and the piece of potato thus prepared is placed upon

* "Wiener med. Jahrbücher," 1883.

† "Beitrag zur Impftuberkulose." "Deutsche med. Wochenschrift," 1883, No. 36.

‡ "Mittheilungen aus dem kaiserl. Gesundheitsamte," 1884, Bd. II, S. 166.

the back part of the tongue, so that it must be swallowed unmasticated. Small cubes of hard-cooked white of egg may be prepared in the same way. The bacteria-material then, to the exclusion of every abrasion of the mucous membrane, passes into the stomach. The previous experiments show that fresh spore-free bacteria, as a rule, are destroyed by the acid of the gastric juice, and do not produce infection. On this account experiments should be made with both spore-free and spore-containing material.

Since infection seems to take place more easily in gastro-intestinal catarrh (at least, according to experiments, a favorable influence on the infection may be brought about by all conditions which directly or indirectly interfere with the acidity of the stomach), the deleterious influence of the gastric juice may be avoided in experiments with bacteria which form no spores, by producing artificially a gastro-intestinal catarrh by drastic cathartics before feeding the animal with the material containing bacteria; or, according to Nicati and Rietsch,* the bacteria may be injected directly into the duodenum. This operation is done, under antiseptic precautions, with sterilized instruments, and with cotton and silk sterilized by steam. The animal, after being tied, is covered with a layer of rubber tissue sterilized by steam. This tissue has an opening only over the spot for the operation. The skin of the abdomen seen through this opening is freed from hair and washed with soap and water over its entire extent, then thoroughly washed with a 1 per mille solution of sublimate, and finally the sublimate is removed by sterilized water. The incision is made in the linea alba somewhat below the xiphoid carti-

* "Semaine méd.," 1884, No. 38. Vergl. auch Deneke, "Deutsche med. Wochenschrift," 1885, No. 3.

lage. After opening the peritonæum, the pyloric end of the stomach is found as quickly as possible and is followed along in the mesentery to the duodenum. This is then fixed with pincettes, and the needle of the sterilized syringe (Pravaz syringe, as modified by Koch) is introduced in the direction of the long diameter of the gut, and the contents of the syringe so injected that nothing passes into the abdominal cavity. (Concerning the construction of the syringe, cfr. *Subcutaneous Injections.*) Then the duodenum is replaced in the abdominal cavity. The wound is united by tight sutures and sprinkled with iodoform. Any further dressing is superfluous.

Since infection is possible not only by the bacteria taken in with the solid food, but also by fluid-nutriment, as water and milk, it is in many cases better to introduce the pure cultures in sterilized water or milk which is used for drinking.

Infection brought about through abrasions is experimentally more at command than that produced by means of the air and food. This method, strictly, ought to be used only for the genuine traumatic infectious diseases. On account of its certainty and availability it has furnished a quite general means for the decision of many pathological questions where the mode of the spontaneous infection is not exactly known.

The Cutaneous Inoculation. — Cutaneous inoculation consists in producing an abrasion of the skin, without injury to the subcutaneous tissue, with a subsequent or simultaneous application of the infecting material. A slight abrasion is made with a sterilized knife on the skin denuded of hair, and upon this superficial wound a particle of the pure culture or of a fluid containing bacteria is placed with a knife or

platinum wire. In place of this operation, performed at two different times, the knife can also be dipped into the pure culture and the incision made directly with the infected knife.

Spots are chosen where the animal can not lick the wound, and which, in addition, permit the course of the process to be followed as much as possible. The ear is especially suitable for this. A cut not drawing blood, or only a few millimetres deep, is made on the inner surface of the ear, not far from the point or on the anterior root in the free skin bordering on the cartilage-rim. In mice, a good cutaneous abrasion can be made almost only at the root of the ear, and even there it is difficult.

The inoculations in the transparent epidermis, which were made by Nassilauf * and Eberth,† can be included here. A superficial puncture is made with an infected needle in the transparent epidermis, and from this puncture bacteria develop in a star-shaped form—the so-called fungous figures.

Subcutaneous Applications.—The subcutaneous application of bacteria is to be distinguished from the true cutaneous inoculation. In a spot denuded of hair, where it can not be licked, a fold of the skin is raised with the pincettes and incised. With a knife or blunt probe a pocket is made under the skin. In this pocket, with a sterilized knife or platinum needle, a particle from a pure culture is placed, and then the skin is again pressed down. In mice, according to Koch,‡ the operation is most conveniently done by seizing the tail of the mouse with long pincettes while

* Virchow's "Archiv," Bd. L, S. 550.

† "Bakterische Mykosen," 1872.

‡ "Die Aetiologie der Milzbrandkrankheit." "Beiträge zur Biologie der Pflanzen," Bd. II, 2. Heft, 1876, S. 279.

it is sitting in a large glass cylinder that is covered, and drawing the tail out of a space between the cover and glass rim not large enough for the mouse to pass, so that it can be well grasped in the left hand. Then, at the root of the tail, in the loose skin, a superficial cross-cut is made with the scissors, and then a pocket, in which a particle of the pure culture is placed with a knife.

Subcutaneous Injections.—For subcutaneous injections a Pravaz syringe, as modified by Koch,* is used, which can be thoroughly sterilized by heat. For this reason it is made without rubber. The metal extremities are united to the glass cylinder by a screw ground in the glass, and the joint is made close by a small cork washer, which is often renewed. The piston of the syringe is wound each time with fresh cotton and silk thread until it fits closely. The syringe, thus prepared, is kept for two hours in a dry-oven at a temperature of 150° to 160° C., and, after cooling, is protected from dust. Before use, the piston is moistened in fresh sterilized, distilled water. For injection, the pure cultures of bacteria, suspended in sterilized water, freshly prepared, are employed.

The introduction of the bacteria into the anterior chamber of the eye is also of importance, especially in dealing with forms which develop so slowly that the first visible effects present themselves in the iris after all the evidences of inflammation have subsided. Cohnheim and Salomonsen† introduced these meth-

* "Mittheilungen," I, 1881, S. 17.

† "Sitzungsberichte der schlesischen Gesellschaft für vaterländische Cultur vom 13. Juli, 1877"; und Salomonsen, "Om Indpodning af Tuberkulose særligt i Kaninens Iris." "Nordiskt Medicinskt Arkiv," 1879, Bd. XI, Nos. 12 u. 19.

ods for tuberculosis. The *technique* is the same as in iridectomy. By means of fixation-pincettes held in the left hand, a fold of the conjunctiva is seized and drawn to the side of the pupil. The other hand introduces, close to the edge of the cornea, a lance, directed obliquely toward the interior. As soon as the point reaches the anterior chamber, the handle is inclined so that the point is directed against the cornea. In this manner also must the knife be withdrawn, as soon as the incision is large enough, so that the iris and lens may not be injured as they press forward, on account of the escape of the fluid from the anterior chamber. Pure cultures can then be thrown in with the syringe, while small particles of material containing bacteria can be introduced with the iris-pincettes.

Direct Injection into the Circulation.—For the direct injection into the circulation the vessel (usually the jugular or crural vein) is exposed with antiseptic precautions, and the needle of the Koch syringe is fastened in the same way as the capillary-tube of Salomonsen (page 123), but so that as little blood as possible flows away. The ligation of the injured vessel, and also the dressing of the wound in the skin, is done in the ordinary way, as in surgery.

If it is not desired, or is impossible, to make inoculations with pure cultures, but the inoculation is to be made from animal to animal, the *technique* is still the same, except that in obtaining material it must be as carefully done, just as if it were desired to use it for the preparation of pure cultures in blood-serum.

If the virulent peculiarities of a form of bacteria have been determined in pure cultures, it is desirable to know approximately the smallest number of the

bacteria which will bring about certain infection; or if the virulence of an infectious fluid is known, the blood, for example, it is desirable to know in what degree of dilution a certain number of drops will surely produce infection. From such experiments an almost direct conclusion may be drawn as to the mode of the natural infection. As yet only quite general principles in this direction have been determined, which show, according to Koch, that the minimum number of bacteria absolutely essential for infection fluctuates, and that this minimum is in relation to the body-weight of the animal. The calculation corresponding to the proposition has been determined for only one form of septicæmia by Davaine,* Koch,† and Gaffky.‡

Bacteria have as yet won but little significance in the pathology of plants, perhaps on account of the general acid reaction of vegetable tissue. Reinke and Berthold# succeeded in infecting a sound potato with a fluid containing bacteria from a damp, decomposing potato. Since the intact skin of a sound potato prevents infection, this must be cut through. For this purpose a three-sided pyramid is cut out with a knife, and into this wound a drop of the fluid or a particle of the pure culture is introduced, and the removed piece is replaced. The inoculated tuber is then placed in a moist chamber. According to Wakker,‖

* "Bulletin de l'académie de medécine," Sitzung vom 17. September, 1872.

† "Untersuchungen über die Aetiologie der Wundinfectionskrankheiten," 1878.

‡ "Experimentelle erzeugte Septicaemie mit Rücksicht auf progressive Virulenz und accommodative Züchtung." "Mittheilungen a. d. kais. Gesundheitsamte," Bd. I, 1881, S. 50.

"Unters. aus dem botan. Laborat. in Göttingen," Heft I.

‖ "Botan. Centralblatt," Bd. XIV, S. 315.

the so-called yellow disease of the hyacinth, observed in Holland, is produced by bacteria which appear as yellow, slimy masses in the vessels of the bulb, and in the vessels and parenchyma of the leaves. The direct proof, that these bacteria were the cause of the disease, was ascertained by transfers to sound plants.

V.

GENERAL BIOLOGICAL PROBLEMS.

After it has been shown by inoculation that the bacteria observed in a decomposition or disease are the cause of this process, it must be determined whether a septic form produces only this one decomposition or fermentation, or whether it acts differently in other media. Thus, e. g., the bacilli of the butyric-acid fermentation produce this form of fermentation in saccharine substances, while in glycerine, according to Fitz, they form propyl alcohol as the chief product.

Further, it is to be noted whether ferment bacteria produce characteristic pigment on certain substances, or whether they may act as pathogenic micro-organisms upon certain animals. According to Brieger, e. g., certain bacilli, which produce proprionic-acid fermentation in saccharine substances, kill guinea-pigs. On this account it is to be determined, by the greatest possible variation in the nutrient media and by the inoculation of different species of animals, whether the septic bacteria, and especially the ferment and pigment bacteria, produce only one single decomposition, or whether they produce different decompositions in different substances; whether they form pigment, and whether they are capable of a parasitic form of life.*

* Cfr. über diese Möglichkeiten: Fitz, "Berichte der deutschen

In the case of parasitic bacteria it is to be determined whether they possess virulent peculiarities only for the one species of animal in which they are spontaneously developed, or whether they are also pathogenic for other animals. The reply to this question is of the greatest importance for the determination of the possibility of the extension of an infectious disease. The pure cultures of the infectious bacteria are to be transferred to different media, since they may possibly decompose a substance in a more typical manner upon which they are able to live. The bacilli of glanders and the vibriones of cholera Asiatica, e. g., produce in certain media a brown pigment, and the cocci of osteo-myelitis call forth lactic-acid fermentation and produce a yellow pigment.

The preparation of the media, and the transfers to these and to animals, is accomplished in the manner previously described. Here it is especially to be noted whether with the change of the medium the form is altered in any way, or whether new forms develop. In the preparation of pure cultures a careful control of the inoculation is to be made as soon as any such peculiarities are observed.

With the aid of pure cultures it is to be determined whether the bacteria are able to free the nitrogen necessary for their growth only from albuminates, and the carbonic acid only from sugar, or whether they can take it up in a simpler union. The compounding of such solutions and the variations of the mineral constituents follow the rule given in the formulæ (Section III, page 94).

Are the bacteria able to peptonize solid albumen

chemischen Gesellschaft," 1882, S. 867; 1884, S. 1188. Und meine Ausgaben in der "Deutsche med. Wochenschrift," 1884, No. 48 bis 50.

and hard-cooked fibrin, or to dissolve coagulated casein?

Casein, freshly precipitated by acid, is washed with distilled water, to remove the acid reaction, and in the same way freshly prepared fibrin is thoroughly washed. It is best to make three parallel experiments, in which, in some test-tubes, only a piece of casein, a flake of fibrin, or a cube of hard-cooked white of egg is introduced with distilled water. In other tubes 0·1 per cent of beef-extract is added, and in a third series of tubes, in addition, 0·5 per cent of grape-sugar is also added. The test-tubes are first thoroughly sterilized by steam, and, after cooling, inoculated, and some are kept at the temperature of the room and others in a culture-oven. Finally, the question of anaërobiosis is to be taken into consideration.

By the inoculation of sterilized milk it is to be determined whether bacteria produce coagulation of the casein by the formation of acid, or whether they separate the casein and then dissolve it after separation.

By the inoculation of milk, sugar, or cane-sugar, it is found that the bacteria make use of double saccharates. Wherefore it is to be noted whether these double saccharates are resolved into the simple saccharates. By transfers to solutions of starch and to starch-paste it is to be determined whether the bacteria dissolve starch and convert it into sugar or not. For these experiments, in some test-tubes nothing is added to the sugar or the starch, in others 1 per cent of beef-extract, and in a third series 1 per cent of beef-extract and 5 per cent of peptone are added.

Enzyme.—All such hydrolytic divisions as are produced by diastase, pepsin, rennet, etc., are, with

tolerable certainty, reduced by the higher organisms to the chemical ferment enzyme, which can be separated from the cells producing the same, and then produce hydration outside of the organism also. If such hydration is produced by bacteria, it is to be determined directly by experiment whether these processes, separable from the bacteria, are real enzyme productions, or whether they can not be separated from the living cells. Hydration * without the presence of a separable enzyme was certainly determined in the lower animals by Krukenberg. I was not able to separate the hydration of milk-sugar by the lactic-acid bacilli from these bacteria, and a recent account by Hansen of a direct alcoholic fermentation of cane-sugar produced by a mould fungus without the separation of invertin is in this sense also significant. There is not the slightest ground for supposing that every hydration brought about by bacteria is without a separable, isolable, true enzyme as the active agent; but concerning this only direct experiments are decisive. The details of these facts belong essentially to the domain of chemistry.† The general process is that the enzyme, with the albuminates, is precipitated by alcohol; these are taken up with glycerine or water, and in this way separated from the albuminates, and this is finally repeated. These procedures must be carried on in sterilized solutions which have been inoculated with pure cultures, and at a time when as yet no spores are present. Weigert ‡ had already

* Kühne, "Erfahrungen und Bemerkungen über Enzyme und Fermente." "Untersuch. des physiologischen Instituts der Universität Heidelberg," Bd. I, Heft 3, 1877.

† Cfr. A. Mayer, "Die Lehre von den chemischen Fermenten," 1882.

‡ "Ueber Glycerin als Unterscheidungsmittel geformter und un-

previously found that glycerine * for this purpose sometimes failed, and I found that alcohol and glycerine were not always reliable for the separation of the enzyme, especially if spores are present.

The separation of the enzyme from fluid containing bacteria may be also attempted by filtration. In the experiments concerning enzyme it is absolutely necessary to determine, by controlling pure cultures, especially plate-cultures, whether bacteria, in spite of all care, were not also transferred.

Ptomaines.—While with the parasitic bacteria, in the narrowest sense, the transfer of the infection is closely united to the transfer of the bacteria, still the action as such, perhaps, may be separable through this fact, that these bacteria form an insoluble enzyme or a basic body similar to an alkaloid—viz., ptomaine—which produces the peculiar poisonous action. By the formation of such poisonous products from the albuminates the putrefactive bacteria themselves may produce intoxication without bringing about a genuine infectious disease. The methods of analyses for such poisons are in part the same as with other enzymes; in part related to the method of the determination of the alkaloids according to the procedure of Stas-Otto; † in part, methods are yet to be worked out, as has recently been done by Brieger.‡

While a greater part of the previously described biological problems can only be solved by the chemi-

geformter Fermente." "Deutsche med. Wochenschrift," 1877, Nos. 40, 41.

* "Mittheilungen aus dem k. Gesundheitsamte," Bd. II, 1884, S. 343.

† Otto, "Anleitung zur Ausmittlung der Gifte," 4te Aufl., 1884. Ludvig, "Medizinische Chemie," 1885.

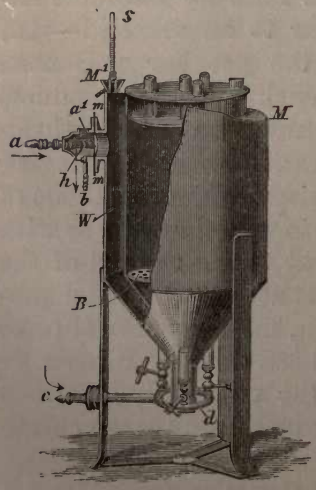
‡ "Ueber Ptomaine," 1885.

cally educated observer, yet a few fundamental questions remain of an essentially experimental character.

BEHAVIOR TO TEMPERATURES.

It is to be determined what is the lowest temperature at which a purely cultivated bacterium begins to multiply and develop into colonies. For this purpose gelatin in test-tubes and sterilized solutions are most advantageously inoculated with pure cultures, and placed for a week in an ice-chest, the temperature of which in general does not fall below 5° C. nor rise above 8° C. Temperatures from 8° C. to the temperature of the room are best attained in cool rooms, in vessels with double walls, through which cold hydrant-water continually circulates; for temperatures from 15° C. to blood temperature, by the insertion of a gas-pressure regulator, the ordinary culture-ovens can be used.

Fig. 28.



For most cases the thermostat, according to d'Arsonval (Fig. 28), is recommended for very exact observations. This consists of a double-walled cylinder made of strong sheet-copper, which ends below in a double-walled conical heating surface. The inclined portion of the cylinder from *M'* to *M* permits the removal of all air and the complete filling of the entire mantle (*W*) with water. The mantle is completely filled to *M'* with distilled or rain water at a tem-

perature approximately near the desired degree. Then the standing-pipe (*S*) is introduced, in which the water rises to a certain height. The gas, independent of the pressure in the remaining pipes, passes through a pressure-regulator, as follows: at *a* it enters through an opening (*a'*) into the small chamber (*h*); from here it goes out again at *b*, and passes from *b* through a tube uniting it to *c*, and then to the burners (*d*). These flames are protected against the draught by chimneys of isinglass and can not be extinguished. Between the room (*h*) and the water-mantle (*W*) a Schloesing's membrane-regulator (*mm*) is extended. In the temperature oscillations of the water in the mantle (*W*) the contraction or expansion of the large mass of water makes itself visible by the relatively greater fall or rise of the water present in the narrow standing-pipe (*S*). In this way the pressure, relatively great, that is resting upon the water is altered, and the rubber membrane (*mm*) is correspondingly drawn, sometimes more toward the water, at others pressed closer to the escape-opening of the gas (*a'*). More gas proportionately passes at times through *a'* into the room, sometimes less, and, as a result of this, the flames burn sometimes higher and again lower, till the temperature is reached at which the apparatus was set.

The regulation to one tenth of a degree is exact for several weeks, if the height of the water in the standing-tube is daily controlled and is regulated by removing a few drops or by adding a few drops of distilled water, and if the gas-pressure is independent of the gas-pressure of the general supply, and the room in which the apparatus stands has an equal temperature. In case of greater temperature oscillations of the room, it is neces-

sary to surround the apparatus with a mantle of asbestos.

With the aid of a thermostat the most favorable temperature can be determined—i. e., that temperature at which the bacteria multiply most rapidly and act most intensely. For this purpose, nutrient gelatin, agar-agar, or blood-serum is inoculated with a pure culture and the time is determined within which distinctly characteristic colonies appear. Then sterilized nutrient solutions in test-tubes and Erlenmeyer's flasks are inoculated with pure cultures, and it is determined by tests, made from time to time, at what temperature the maximum of the action—e. g., a certain degree of acidity—is attained in the shortest time. The temperature most favorable for multiplication and action is restricted sometimes within a few degrees, but often also shows great latitude. Beyond this point ensues again a diminution of the rapidity of the growth and intensity of the action. For the determination of these limits, pure cultures are inoculated both in solid media and in favorable fluids—e. g., thus it is determined that the multiplication and the action of the lactic-acid bacteria cease between 45·3° C. and 45·5° C. in milk and 3 to 4 per cent sugar solutions, without the death of the bacteria ensuing. These limits indicate at first only a cessation of development, a kind of fixation by heat. Beyond these the first real destruction of the bacteria ensues.

After Pasteur, in February, 1880, had made the discovery that by biological interference the virulence of chicken-cholera was diminished, and that inoculation with this attenuated virus protected the animals against inoculation with virulent material, Toussaint found that virulent anthrax-blood, by warming ten

minutes at 55° C., or by the addition of carbolic acid, was rendered less virulent, and Pasteur, a little later, found that by cultivating the pure anthrax-bacilli in bouillon at 42° to 43° C. the virulence of this bacterium could be systematically attenuated; then Chauveau * discovered that at 52° C. fifteen minutes, at 50° C. twenty minutes, at 47° C. three or four hours, at 43° C. about six days, and at 42° C. nearly twenty days were necessary to obtain the same degree of attenuation, but that the certainty and the constancy of attenuation were the greater the lower the temperature. Koch, Gaffky, and Löffler † made these observations for the temperature between 42° and 43° C. still more precise and exact.

Such fine gradations of the temperature can only be obtained with an excellent thermostat. In a similar manner it is to be determined at what high or low temperatures spores form, and below and above what temperatures no spores are formed. Within these limits it is to be determined what is the most favorable temperature for the formation of spores, so that the number of the spores and the regularity of their formation may be observed in cultures, and the time ascertained at which the first spores appear. Also the time necessary for the germination is to be determined at different degrees of temperature. These deductions concerning the lowest temperature at which a form of bacteria may still multiply, concerning the height and extent of the most favorable temperature, and concerning the high temperature at which a form does no longer act; further, the observations concerning the high and low

* "Comptes rendus," T. 96, 1883, Nos. 9, 10, 11.

† "Mittheilungen aus dem k. Gesundheitsamte," Bd. II, 1884, S. 147.

temperatures at which the formation of spores no longer occurs, and the deduction concerning the most favorable temperature for spore-formation, and the temperature necessary for spore-germination—almost alone permit the correct conclusion as to the degree of the adaptability to the parasitic mode of life.

If the difficulty or the ease of the preparation of the pure cultures is taken into consideration; if it is noted whether the purely cultivated bacteria, in respect to their need of nitrogen and carbonic acid, are very dainty or not; if the favorable conditions of temperature described are tested—then the pure culture is under good conditions for an epidemiological experiment. The experimenter can by these inquiries protect himself against the one-sided, contagious significance of the interpretation of the experiments on animals, and can come unprejudiced to the review of the local and temporary auxiliary causes for the origin of an epidemic or endemic disease.

In order to determine more exactly the temperature at which spores are killed, it is best to proceed by using a deep water-bath, which has a constant level of the water. Upon the bottom of the water-bath a wooden plate is placed to protect against the direct action of the hot bottom. Above, the bath is closed with a cover which has a number of openings for the introduction of several thermometers, extending to different depths, and for the reception of test-tubes containing sterilized nutrient solutions. The water of the water-bath must stand higher than the plane of the fluid in the tubes, in which the temperature remains somewhat lower than that in the water-bath, so that the indicated degree does not correspond exactly to the temperature really obtained in the test-tubes. When an equalization of the temperature has been obtained, after about

half an hour, the tubes, having been opened, are rapidly inoculated with spores, and repeatedly moved to and fro. The tubes are left at different temperatures —e. g., boiling temperature, 95° C., and from 90° to 70° C. for different lengths of time; then a few test-tubes are placed at the temperature most favorable to the development of the form under consideration, in order that it may be determined, by the appearance or non-appearance of cloudiness, as to the death of the germs. Gelatin may be mingled with the contents of other tubes, and these used for plate-cultures. In the study of the effects of temperature above 100° C., the salt- or oil-bath is best used for the experiments.

The spores, also, before being killed, may be attenuated, as is shown by transfers to suitable solutions or animals. In order to determine the influence of dry heat, short pieces of silk thread are soaked in a fluid free from spores, and in another containing spores, by laying the threads in a suspension of a pure culture of the corresponding bacteria in distilled water, or by placing them in fresh blood containing bacteria, or in the tissue-fluid. Then the threads are dried in an exsiccator, laid in a watch-glass, and directly exposed in a dry-oven to different elevations of temperature.

According to the kind of action of the temperature, as mentioned, a simple checking of development, or an attenuation of virulence or destruction of the bacteria, is made perceptible.

Besides temperature, many agents act according to concentration in preventing development, as antiseptics, or attenuating or killing the micro-organisms, as disinfectants.*

* Koch, "Ueber Disinfection." "Mittheilungen aus dem k. Gesundheitsamte," 1881, Bd. I, S. 234.

DISINFECTION WITH FLUIDS.

Experiments concerning disinfection with fluids are made by filling watch-glasses, crystallization-dishes, or test-tubes, with a solution of the agent to be tested in different degrees of concentration, and by introducing into some of these threads of silk impregnated with bacteria and spores, and into others threads containing bacteria alone.

After different lengths of exposure the threads are removed from the solution with a sterilized pipette, washed with sterilized water, and three or four threads are placed in a thick fluid nutrient gelatin, which is laid on a slide, or in other tested nutrient media. For control, threads which have not been treated at all are placed in gelatin, and others which have only been washed with sterilized water. The difference in the time of development, or the complete non-appearance of development, can then be directly observed. Further control is necessary by introducing such silk threads under the skin of animals. Carbolic acid, e. g., kills the anthrax-bacilli in a 1 per cent solution in two minutes, while it requires seven days for a 3 per cent solution to destroy their spores. The development of the anthrax-spores is prevented by exposure to a 2 per cent solution for three days.

Chamberland and Roux * inquired into the attenuating influence of carbolic acid by adding a definite amount of the agent to neutralized calf-bouillon which was inoculated with anthrax-bacilli. Carbolic acid, 1 to 400, prevented development and destroyed the bacilli in forty-eight hours; 1 to 500 killed them after five months; 1 to 600 only after six months.

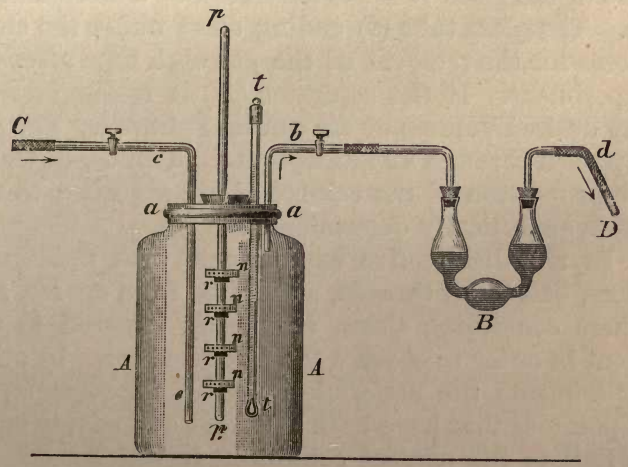
* "Comptes rendus," Bd. XCVI, 1883, No. 15.

In 1 to 600 the bacilli, after twelve days, killed only guinea-pigs, rabbits, and mice, and after twenty-nine days were inactive. The addition of carbolic acid in the ratio 1 to 800 prevented the formation of spores, which first appeared when the proportion was reduced to 1 to 1,200.

DISINFECTION WITH GASES.

The following arrangement of the apparatus after Fischer and Proskauer * is recommended for laboratory experiments on disinfection with gases. A thick-walled glass vessel (*A A*) of about 20 litres capacity is closed with a strong cover (*aa*) of vulcanized rubber, the rim of which is pressed tight upon the neck of the vessel by means of a narrow ring. In the cen-

FIG. 29.



ter of the cover is a large perforation, and around about this several smaller ones, which can be closed

* "Ueber die Disinfection mit Chlor. und Brom." "Mittheilungen aus dem k. Gesundheitsamte," Bd. II, 1884, S. 228.

simultaneously by rubber stoppers. The central perforation, 5 to 6 cm. broad, is for the admission of the objects to be used for the experiment, which, arranged so that the gases may have entrance on all sides, are placed on sieve-like, perforated paraffine dishes (*nn*). These dishes, whose diameter for easy introduction is somewhat less than the central perforation, are fastened to a glass rod (*pp*) which passes through their center so that several such dishes can be arranged one above the other. These are held in place on the glass rod by means of rubber rings (*rr*). The glass rod itself is placed firmly in a rubber stopper which exactly closes the large central opening. The glass tube (*cc*), that reaches almost to the bottom of the vessel and is bent at a right angle, conducts into the glass vessel the gas developed in the apparatus (*C*). The temperature of the glass vessel is measured by a thermometer (*t*). The glass tube (*b*) ending close under the cover serves for the removal of the gas each time after the experiment. If the aspirator (*D*) is set in motion, any desired volume of air can be removed. The gas is passed through an absorption-fluid in *B*, in which the proportion of the volume of air absorbed to the gas is analytically determined.

In the disinfective experiments with fluids and gases, dried silk threads, some free from spores, and others containing them, are freely exposed to the agent in order to observe the direct effects. In other experiments the material is packed in filter-paper, in potatoes that have been hollowed out, etc., in order to imitate the natural conditions in which access to the germs is more difficult.

DRYING.

In order to study the influence of drying, a drop of culture-fluid containing bacteria is placed upon a cover-glass and protected from dust, dried, and, after an hour, a day, or a week, a drop of culture-fluid is placed upon the dried preparation, and the cover-glass with the hanging-drop is transferred to a moist chamber. The exhaustion of the nutrient fluid brought about by the drying under otherwise favorable conditions becomes perceptible in the anthrax-bacilli by the development of spores in the filaments, which after months, on the addition of nutrient solutions, again germinate. In the cholera vibrione, on the contrary, no spores form, but, as an indication of the exhaustion of the nutrient medium, a spirilla-like thread-formation appears. On this account, the drying, after a short time, also produces death, so that later, on the addition of nutrient media, no development of these bacteria occurs.

The parasitic bacteria, attenuated directly or in their spores, may perhaps afford protection against the virulent bacteria. If the animals used for experiment have withstood the inoculation of the attenuated material, they are infected after a longer or shorter interval with virulent material in the same manner.

THE ACTION OF LOW TEMPERATURE AND HIGH PRESSURE.

The influence of low temperature and high pressure upon bacteria can only be studied with a special apparatus. The previous statements as to this are so widely at variance that I shall content myself by merely alluding to this subject. It is also desirable

that this phase of the method should be somewhat better studied, because of the statement of the existence of aërobic bacteria at considerable depths in the sea, and of the resistance of the bacteria to a pressure * of 1,000 atmospheres artificially produced. Here also belong the declarations of Chauveau and Wossnessenski,† that an increase of pressure produces first an attenuating influence, and at a certain degree causes death in the anthrax-bacilli. While the resistance of many bacteria to frost at a moderate freezing temperature has been certainly determined, and can be easily proved by exposure of the inoculated solution or the pure culture in winter, yet statements vary considerably as to the most extreme temperature below zero, C. Pictet and Yung ‡ place as the lower limit 108 hours at 70° C., and 20 hours at 130° C.

ELECTRICITY.

According to Cohn and Mendelsohn,# electricity in the form of a physiologically acting induction-current is without influence upon bacteria in fluids.

The action of the constant current upon the multiplication of bacteria in nutrient solutions, and also upon the development of the micrococcus prodigiosus upon the surface of potatoes, depends upon the strength of the current, and was referred to its electrolytic action.

PHOSPHORESCENCE.

The phosphorescence of bacteria Ludwig ‖ has endeavored to study by a micro-spectral apparatus.

* Certes, "Comptes rendus," Bd. 98, S. 690 und 745; Bd. 99, S. 385. † *Ibid.*, Bd. 98, S. 314. ‡ *Ibid.*, Bd. 98, S. 747.

"Beiträge zur Biologie," Bd. III, Heft 1, S. 141.

‖ "Ueber spectroskopische Untersuchung photogener Pilze." "Zeitschrift für wissenschaftl. Mikroskopie," 1884, Bd. I, S. 181.

LIGHT.

Engelmann* found that the swarm movements of the bacterium photo-metricum are dependent upon light, and that the collection of cells in the sun-spectrum is most marked in ultra-red, and decreases more and more through the green and blue to the violet.

* "Arch. f. d. ges. Physiologie," Bd. 30, S. 95.

VI.

SPECIAL HYGIENIC INVESTIGATION.

A. EARTH.

It was first experimentally determined by Schlösing and Müntz * that the ammonia formed in earth from the disintegration of organic substances can be reduced by the action of bacteria to nitric acid. The oxidation of the carbon of organic substances to carbonic acid was likewise experimentally proved by Vollny † to be due to micro-organisms. On the contrary, there occurs in layers of earth restricted from the entrance of air a reduction of the nitrates, as Guyon and Dupetit ‡ found, in which sometimes only the nitrites, but at others nitrogen, ammonia, and nitrous oxide are formed by the action of bacteria. These experiments are performed by adding a particle of earth to a weak alkaline nutrient solution, or to drain-water which has been sterilized and contains the ammoniac salts, nitrites or nitrates, while for control a particle is placed in another glass which has been sterilized by many hours' heating at 150° or 160° C. For bringing about the reduction of the nitrates the arrangement of experiments employed in anaërobiosis is used.

* "Comptes rendus," Bd. LXXVII, 1877, S. 203 und 353; Bd. LXXXIV, S. 301; Bd. LXXXIX, S. 891.

† Landw., "Versuchsstationen," Bd. XXV, 1880, S. 390.

‡ "Comptes rendus," 1882, Bd. XCV, S. 644 und S. 1365.

By this were furnished the first positive grounds for the biological explanation of the soil-water theory of disease, which had previously been purely hypothetical.

The direct determination of the presence of pathogenic organisms, or their spores, in the earth was made in 1881 by Pasteur and Koch* for the bacilli of malignant œdema. Nicolai† cultivated a form of bacillus from the earth which produced in mice, rabbits, and guinea-pigs a fatal form of tetanus, which was transferable to other animals.

Such inquiries make it seem desirable that a more exact study of the bacteria present in the earth should be made, because in it optional parasitic bacteria find, with sufficient moisture and temperature, the conditions necessary for existence, while the optional septic bacteria can thrive in it, at least for a time. The obligatory parasitic bacteria can at least be preserved in the form of permanent spores in the earth.

In order to form the most comprehensive conclusion possible as to the presence in the earth of bacteria or germs of the same capable of development, the gelatin-plate cultures of Koch are most advantageously used, because of reasons previously stated, in which collectively the septic bacteria and the optional parasites at present known and the optional septic bacteria can develop into isolated colonies. Since the individual organisms already found in the earth, as the bacilli of malignant œdema and of butyric-acid fermentation, are anaërobic, the gelatin-plates are covered with sheets of mica. It is to be observed also whether the species develop from spores,

* "Mittheilungen aus dem k. Gesundheitsamte," Bd. I, 1881, S. 56.

† "Ueber infectiösen Tetanus." "Deutsche med. Wochenschrift," 1884, No. 52.

and whether at a certain depth only the bacteria-forming spores, are met with. The earth to be used in the experiment is pulverized in a sterilized mortar, with a sterilized pestle, and with a sterilized scalpel; some of the fine powder is scattered upon the still partially fluid gelatin, which has been poured out upon a plate.

A few fine particles of the earth may also be introduced into the liquefied gelatin while still in the test-tube, and thoroughly mingled by shaking, and then the gelatin thus infected may be poured upon a plate. The control is made by preparing plates in the same way from particles of earth which have been sterilized by two hours' heating at 150° to 160° C.

Strongly obligatory parasitic bacteria can be present in the earth only in the form of permanent spores, and these can be directly cultivated from the earth only in a special manner. As a rule, it is necessary to use the round-about way of the infection-method, in order to procure an original material as clean as possible for the blood-serum cultures. Particles of the earth are placed in one or more pockets beneath the skin, and then another animal is infected with the pus, blood, or tissue-fluid of the sick animal. After a few transfers the animals will present in their tissues pure cultures of the malignant form of bacteria, and then blood-serum cultures can be prepared in the previously described manner, as was successfully done by Nicolai.

B. WATER.

The chemical investigation of water alone can furnish no information as to the hygienic fitness or unfitness of a certain water for use and drinking. The water chemically purest—the distilled water of the

laboratory, for example—always contains bacteria and their germs; and, on the other hand, water tolerably dirty chemically may contain relatively few micro-organisms. Chloride of sodium, the type of the chemical substances from inhabited places, always presents a valuable index of the cleanliness as compared with good spring-water, and in the same way the nitrites are significant, because they afford information (from the previously described reasons) that the water is in relation with localities in which the biological processes have not yet come to a rest, since the nitrites are produced by oxidation or reduction. With freedom from its limitations and with careful consideration as to the local conditions, now as formerly, valuable conclusions may be drawn as to the hygienic value of the water from the usual chemical examination.

But not only the soluble matters of the biological processes in the earth may be mingled with the water, but also the organisms themselves. *A priori*, there exists not the slightest ground for attributing to the soil-water alone, or rather to the moistening of the subsoil caused by the same, an equal importance in the existence and extension of infectious diseases with the water for use and drinking. The publication of localized investigations has added information to both possibilities.

The direct microscopical examination of water is made both in the hanging-drop and in the dried cover-glass preparations. By culture-experiments, on the one hand, the number of germs capable of development is determined, and on the other the presence or absence of pathogenic species among them.

The water is collected in sterilized vessels. Then 1 c. cm. is drawn into a sterilized pipette and thor-

oughly mixed in a test-tube with about 10 c. cm. of a 10 per cent nutrient gelatin liquefied at 30 C. This mixture is poured out upon a sterilized glass plate, which, after the solidification of the gelatin, is placed in a moist jar.

In these plate-cultures the strongly obligatory parasitic bacteria can not develop, and there are rarely so many germs present that they can not be numbered. Notwithstanding that the obligatory parasitic bacteria do not develop in plate-cultures, these are for hygienic purposes of equal value, because it is of the first importance in the foundation or improvement of a water-supply to know whether the water contains pathogenic micro-organisms which can exist outside of the body.

If the water is very rich in germs, it must be diluted in a definite manner with 10, 50, or 100 to 1,000 c. cm. of sterilized distilled water. On practical grounds, in order to determine a directly comparable number, it is recommended by Koch* that the number of germs capable of development in 1 c. cm. of the original water be computed.

By the form of their development, suspected or unknown species are brought to a proof as to their malignant peculiarities by inoculation of animals.

If the water can be directly employed for examination, it is best to fill sterilized flasks, which are immediately closed with sterilized cotton stoppers. For transportation the following procedure is recommended: Small cylindrical bottles, of about 100 c. cm. capacity, provided with ground-glass stoppers, are thoroughly cleaned in the laboratory and

* Cfr. auch Becker's "Anleitung zur Untersuchung des Wassers auf Mikroorganismen." Börner's "Reichs-Medizinal Kalendar für 1885," Heft II.

sterilized at 150° to 160° C. Then a rubber cap, sterilized by steam, is closely drawn over the stopper, and the vessel, with the cap, is inclosed in a small wooden box. For the reception of water from a supply-pipe, after the water has first been allowed to run for a few minutes, the vessel is filled under the faucet, while the rubber cap and the stopper are held with sterilized fingers. Then the stopper is replaced, the cap is drawn over, and the vessel is inclosed in the box.

For obtaining water from a spring the rubber cap is removed, and the glass stopper, withdrawn under water, is replaced again after a minute or two; the cap is again drawn over it, and the vessel is dried on the outside and placed in the box. In this manner any infection outside of the water is prevented.

The number of colonies developing is not always equal, especially as every elevation of the temperature is associated with an increase in number of the germs. Standing for one day in a warm room has an influence in this way. In the same way as the number of germs which develop varies, the number in the different forms also varies considerably. The judgment in the bacteriological side of water investigation presupposes previous studies in bacteriology.

C. AIR.

In order to determine the germs present in the air, Pasteur caused the air to pass through gun-cotton; then dissolved the gun-cotton in ether, and examined the solution microscopically. Miquel * endeavored, with improvements of the previous apparatus, to number the micro-organisms directly. In a jar, with the opening looking downward, a hollow

* "Annuaire de l'observatoire de Montsouris."

ball is screwed, which has a fine opening at the apex. Opposite to this opening in the jar a cover-glass is placed, which is smeared with glycerine and sugar. If the air is aspirated into the jar, it must first strike upon the cover-glass and surrender its dust to the sticky fluid, which is then microscopically examined. For numbering the bacteria, Miquel causes a measured volume of air to pass through a sterilized, neutralized solution of beef-extract.

Emmerich * devised an improvement of the aëroscope for fluids. A pear-formed glass (Fig. 30) communicates by a fine opening with a glass tube, 70 or 80 cm. long, which leads to a glass ball by about seven gently rising, parallel spirals. The apparatus is filled with 20 or 25 c. cm. of a nutrient solution, and the air is drawn through by aspiration, which, in small bubbles from the opening for entrance, passes out by the long way through the spiral, so that it comes in intimate contact with the solution, and, on this account, can easily surrender its germs to it.

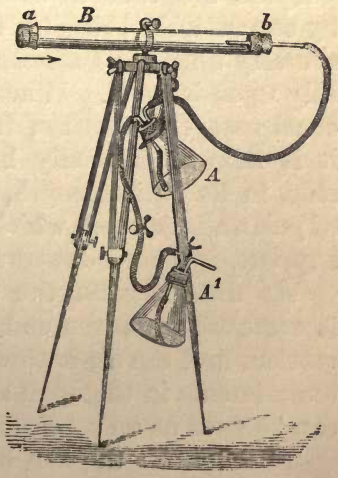
FIG. 30.

Since, also, in the complete absorption of the germs by the sterilized nutrient solution, serving as a wash-fluid, an isolated development of the germs, and thus a direct enumeration, is impossible, the practicability of all aëroscopes with the use of fluid ceases where really a beginning should be made. The possibility of testing a fluid laden with germs as to its contents in germs is rendered practicable by mingling these solutions with sterilized nutrient gelatin, so that then the individual germs isolated can develop into colonies in the gelatin.

* "Archiv für Hygiene," Bd. I, 1883, S. 169.

After Koch * had demonstrated in principle the value of nutrient gelatin for the examination of air also, Hesse † constructed the following apparatus, which consists of a long glass tube and an aspirator. The glass tube (*B*, Fig. 31) has a length of about 70 cm. and a diameter of 3½ cm. The end (*a*) is closed by a closely fitting rubber cap, provided with a central round perforation, and over this a second similar cap is placed, which has no perforation, and, on this account, completely closes the end of the tube. The end (*b*) is provided with a tightly fitting rubber stopper, in the center of which is a hole about 1 cm. wide. Through this a glass tube, about 10 cm. long, is introduced, the free end of which is connected with the aspirator (*A* and *A'*). In this glass tube two cotton stoppers are placed, one of which lies near the center, while the other projects inward somewhat into the lumen of the tube.

FIG. 31.



After removal of the rubber stopper the tube is filled with about 50 cm. of a sterilized nutrient gelatin (containing 5 to 10 per cent of gelatin), the end is closed again, and the tube with the gelatin placed

* "Mittheilungen aus dem kaiserlichen Gesundheitsamte," Bd. I, 1881, S. 32.

† "Ueber quantitative Bestimmung der in der Luft enthaltenen Mikroorganismen." "Mittheilungen," Bd. II, 1884, S. 182.

for one to two hours in hot steam. After the tube has cooled enough, so that it can be handled readily, the entire inner surface of the tube to *B* is coated with a thin layer of gelatin, while the bottom, upon which the germs will almost exclusively settle, is clothed with a greater mass of gelatin. For this purpose the tube is cooled under the water-faucet by successively bringing it in its entire length horizontally under the stream, and then rotating it rapidly upon its axis. When the gelatin becomes quite consistent the rotation is immediately ceased, and it is then moved only horizontally. Thus on one side, in its entire length, a somewhat thicker layer of gelatin is formed, which, in putting the tube on its support, is placed downward as the floor layer.

At the place for the observation the outer cap is removed from the end (*a*), the aspirator is set in motion, and the air is slowly drawn through. In the experiments in the open air two separated litre-flasks can be used as an aspirator.

In order, on the one hand, to meet the objection that not all the germs cling to the surface of the gelatin, but especially for the improvisation of an apparatus which unites the advantages of the gelatinizing substances with a wash-fluid, recourse may be had to the following procedure: Sterilized test-tubes are provided with rubber stoppers that have been sterilized in steam, and in which are two perforations. In both of these, sterilized glass tubes, bent at right angles, are introduced; one of the tubes ends directly underneath the stopper, while the other passes almost to the bottom. The test-tubes, thus armed, are filled with nutrient gelatin, or, according to von Sehlen,* with agar-agar which has been carefully sterilized. The

* "Studien über Malaria." "Fortschritte der Medizin," 1884, No. 18.

free ends of the glass tubes are closed by sterilized cotton stoppers, which are removed at the spot from which the air is to be taken. The gelatin is liquefied by placing it in water at 30° C., the agar-agar at 40° C., and during the aspiration it is held in solution at this temperature, so that, as a nutrient solution, it receives the germs.

Care must be taken to join two or three such test-tubes with one another, since if a large amount of air is drawn in, the first tube will not contain all the germs of the air. After completion of the experiment the cotton stoppers are replaced and the solutions solidified by removing them from the warm bath. In order to prevent, in these cases, any further infection, the germs are allowed to develop isolated in the test-tubes.

VII.

BACTERIOLOGY AS AN OBJECT OF INSTRUCTION.

If the methods thus far developed for the investigation of bacteria should obtain the significance which the mass of physicians ascribe to these things, then our high-schools must furnish the opportunity for instruction in this important subject.

Up to this time this has been done in a quite insufficient manner, from the lack of suitable instructors and institutes. Great praise is due the University of Copenhagen for going in advance, breaking the way, and for having established in 1883, in connection with the Botanical Institute, an institute for medical bacteriology, the direction of which was discreetly placed in the experienced hands of Salomonsen. Of the German universities, as yet only Munich in connection with the pathological institute, Göttingen in the hygienic, and Breslau in the botanical institute, furnish the opportunity for learning the methods. In addition to these there is still the hygienic bacteriological department of the chemical institute of Fresenius at Wiesbaden, directed by myself, which takes teaching into special consideration. The laboratory of the office of the Imperial Board of Health is only accessible in a civil and diplomatic way, so that for the general purpose of teaching it does not come at all into consideration.

The zeal with which the work on this subject is now done at all high-schools would be exceedingly fruitful, and would render many a fiasco impossible, if an opportunity for work in the methods were afforded in a manner sufficient for the existing needs. The demand, as such institutes are constituted, is at the moment scarcely to be answered in a sufficient manner, from the want of suitable instruction and from pecuniary considerations.

Botany is interested in the solution of many morphological and biological questions in bacteriology. (But a distinguished botanist has sorrowfully complained of the disgrace that most botanists do not concern themselves with bacteriology.) These questions can be referred to the botanical institutes, of which there is no lack.

It is impossible for physiology to be satisfied with the present condition of the subject of digestion, but it must now give quite a different consideration than formerly to the biological processes in the intestines. The solution of these problems, which are associated in part with the physiology of fermentation, can be referred exclusively to the richly endowed physiological and physiologico-chemical institutes.

Also pathology, sufficiently provided with institutes among the many attractions with which it has to do, can not fail to give greater consideration to bacteria as a further field for investigation.

Whoever has really interested himself in bacteria investigation is rejoiced when the subject is undertaken from the most different standpoints. But such divergent standpoints do not suffice for the investigation, if they do not succeed in giving to bacteriology its due place as an object of study.

For a definite basis for bacteriology it seems to me

that only two ways offer a prospect for lasting results: first, special bacteriological institutes can be founded, as at Copenhagen; or, secondly, these institutes ought to be united with the hygienic institutes to be erected. The latter seems to me to be the most promising plan.

Botany, physiology, and pathology have always an actual interest only on a certain side of the investigation, while to the other questions they are placed in nearly equal relation.

The botanical questions possess an important interest for hygiene, because the formation in the system, the constancy and the variability of forms, etc., can have a direct influence upon the hygienic treatment. Hygiene shares this interest in botanical questions in part with physiology and pathology. Hygiene must know the general biology of bacteria, because the general processes of decomposition brought about by septic micro-organisms are of significance in the consideration of the ætiology of disease. Botany and physiology in part share in this interest.

Hygiene must be very familiar with the pathogenic bacteria, since these have not only the value perhaps of an external attraction for the ætiology, but because, as the *conditio sine qua non*, they form the hinge of the entire ætiology, which pathology can consider only in an insufficient manner, because the processes in the body lay claim sufficiently to their activity.

The problems of combining the experimental investigations concerning the parasites of disease with the results of the investigations as to the accessory causes of epidemic diseases (basing the hypothesis of the soil and drinking-water theories upon the biology of

micro-organisms and facts, and no longer exclusively upon reflections and probabilities) belong only to hygiene, and are to be solved in a satisfactory manner only by looking well to all sides of the investigation, few parts of which are, after all, only sufficiently recognized by botany, physiology, and pathology. It seems to me to follow, from these considerations, that that branch of science which from its very nature must be accountable to the whole field is best adapted in the fullest sense to do justice to the subject in hand as an object of teaching.

In the same manner as the teacher of hygiene, in another direction, is required to familiarize himself with bacteriology to enable him to impart the same to his pupils, so must we also require of the bacteriologist intrusted with hygiene that he should master the other chapters of hygiene to become a teacher.

Hygiene can not and must not be lost sight of in the importance of bacteriology, and it would be a sad error to consider only the bacteriological side in filling the chair of hygiene. Bacteriology as an object of study, as seen in some institutions, would be more practical if combined with hygiene.

It is self-evident that, in the methods of teaching, two things must be kept well separated—i. e., the introduction into the methods by practical courses, and independent working after preparatory instruction; the latter will only be employed by a few especially interested, but it must be sufficiently observed in the equipment of the institution. The actual every-day necessities lie at present more in the preparatory courses. Every practicing physician, and especially every medical officer, is desirous of so far informing himself concerning these methods as to be able to follow intelligently the recent investigations, since the

morphological and biological foundations of the general processes of decomposition and the ætiology of infectious diseases have assumed tangible form through the development of the methods.

Courses may be arranged in the form of practical exercises lasting from four to six weeks, in which the most important methods are practiced with particular regard to those most important, considered from a hygienic standpoint. This plan alone might at the time be adequate to meet the necessities of medical officers and any surgeons who can absent themselves from duty for only a few weeks, but is far less agreeable to the teacher than if he could distribute the practical introduction into the methods over a semester. The latter plan, which must become the rule for the young medical man and the naturalist, permits the teacher to take cognizance of all methods, and thus to render the perception more acute for the development and for the future problems of investigations.

As a result, this mode of introduction into the methods becomes an important aid, which well prepares for independent work, and may bring about the entire disappearance of over-hasty publications in bacteriology.

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